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Evaluation of cytotoxic Effect, anticholinesterase, antioxidant, antiarthritic and antibacterial activities of the Algerian species *Scabiosa stellata* L.

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Abstract: The present study investigates the evaluation of *in vitro* pharmacological activities of crude extracts (petroleum ether, ethyl acetate and *n*-butanol) obtained from the plant *Scabiosa stellata* L. The cytotoxicity of extracts was tested by Brine shrimp lethality method; the acetylcholinesterase inhibitory activity was performed using Ellman's colorimetric method. The anti-arthritic activity was conducted by bovine serum albumin denaturation method, and the antioxidant activity was evaluated by six different methods including DPPH and ABTS radicals scavenging activities, reducing power, CUPRAC assay, ferrous ions and metal chelating assay. Furthermore, the antibacterial activity was estimated by agar disk diffusion assay against ten bacterial strains. The phytochemical screening of all the extracts revealed the presence of several types of secondary metabolites. The ethyl acetate extract recorded the highest content of polyphenols, flavonoids and tannins. All the crude extracts (PE, EtAOc and *n*-BuOH) had antioxidant activities in various assays and prevent the denaturation of bovin serum albumin in dose depending manner. A significant cytotoxic effect was observed for the *n*-butanolic extract with 57.2 ± 0.2% of mortality at 80 µg/mL, the ethyl acetate extract displayed a moderate anticholinesterase activity at 200 µg/mL. All the crude extracts showed antibacterial activity against most tested strains, with zones of inhibition ranging from 9 to 20 mm. The results indicate that the species *S. stellata* could be an important source of therapeutic agents against neurodegenerative inflammatory and infectious diseases.

Keywords: *Scabiosa stellata* L., Cytotoxicity, Anticholinesterase, Antioxidant, Anti-arthritic

Introduction

The supplementation of the body by exogenous bioactive compounds is one of the new therapeutic strategies to prevent the appearance of cancers oxidative, inflammatory, infectious and neurodegenerative diseases [1-2]. In this context, many researchers are interested in medicinal plants as an alternative and important source of natural compounds to find new antioxidant and anticancer agents with few side effects, which act according to several modes of action to reduce the effects of free radicals and the appearance of oxidative stress-related diseases (cancers, neurodegenerative and inflammation). These species are found in many families.

The family Dipsacaceae is represented by three tribes and comprises nine genera.The genus *Scabiosa* comprises about 100 species [3]. Plants of this genus are widely used in traditional medicine for the treatment of various

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diseases including hypoglycemia [4], respiratory diseases and menstrual regulation [5] and for their diuretic effects [6]. They are also recommended in dermatoses and against ulcers [7]. *Scabiosa stellata* L. is an annual plant with bluish flowers, distributed in North Africa [8] and used in Moroccan popular medicine against heel cracks [9] and for the treatment of various respiratory diseases including bronchitis, bronchial pneumonia, influenza and asthma [10].

The present study investigated the biological evolution of crude extracts (petroleum ether, ethyl acetate and *n*-butanol) obtained from the plant *Scabiosa stellata* by the estimation of total phenolic and flavonoid contents and the assessment of its *in vitro* cytotoxic, anticholinesterase, antioxidant, antiarthritic and antibacterial activities.

Material and Methods

Plant material

The plant *Scabiosa stellata* L. was collected in May 2014 in the Aures region (Bellezma, Algeria) and was identified by Professor Bachir Oudjehih, Agronomic Institute of the University of Batna-1, under the number 2224/LCCE.

Preparation of plant extract

The powder of the whole plant *Scabiosa stellata* (500 g) was macerated twice (5 Lx 2 each 48h) with a solvent mixture of ethanol- H2O (70:30) at room temperature. After filtration, the filtrate was concentrated under vacuum at room temperature to obtain a hydro-alcoholic extract (400 mL). This solution was submitted to liquid-liquid fractioning using organic solvents PE, EtOAc and *n*-BuOH successively (100 mLx 5 of each organic extract) until the separation of both phases aqueous and organic. The obtained phases were dried over anhydrous sodium sulfate, filtered and evaporated to to provide dryness extracts PE (1.03 g), EtOAc (6.52 g) and *n*-BuOH (30.38 g).

Phytochemical Screening

In order to detect the different secondary metabolites present in crude extracts of *Scabiosa stellata*, a phytochemical screening was carried out using the methods described by Fransworth (1966) [11] based on the observation of color changes in the initial mixture or the formation of a precipitate.

Total Bioactive Content

The quantification of total phenolic content of the methanolic extract was assessed spectrophotometrically using the Folin-Ciocalteu method [12]. 200 µL of the sample was added to 1 mL of Folin-Ciolcalteu solution (10 %). After 4 minutes, a volume of 800 µL of sodium carbonate solution (Na_2CO_3, 75 g/L) was added. The mixture was incubated for two hours in darkness at room temperature. After incubation, the absorbance was read at 765 nm using UV-Vis spectrophotometer (VIS-7220G). Gallic acid (50-500 µg/mL) was used as a standard to establish the calibration curve from which the concentration of polyphenols was calculated and the results were expressed in microgram equivalents of gallic acid per milligram of extract (µg GA/mg of extract).

The total flavonoid content of MeOH extract from *Nonea vesicaria* was performed by the trichloroaluminum method [13]. 1 mL of trichloroaluminum solution prepared in water ($AlCl_3$, 2 %) was added to 1 mL of the crude extract. The mixture was vigorously agitated and incubated for 10 minutes at room temperature and then the absorbance of the sample was measured at 430 nm. Quercetin (25 - 200 µg/mL) was used to realize the calibration curve to determine the concentration of flavonoids and the results were expressed in microgram equivalents of quercetin per milligram of extract (µg QE/mg of extract).

The analysis of condensed tannins was processed using the method described by Heimler et al [14]. 3 mL of vanillin solution (4 %) were added to 50 µL of tested extracts and then 1.5 mL of concentrated hydrochloric acid were added to the mixture. After 15 min of incubation, the absorbance was measured at 500 nm. Catechin (10 - 300 µg/mL) was used to establish the calibration curve to calculate the concentration of condensed tannins in the extracts. The results were expressed in micrograms equivalent of catechin per milligram of extract (µg EC/ mg extract).

Antioxidant Activities

DPPH free radical-scavenging assay

The antioxidant activity of extracts was evaluated using the method of Blois et al [15]. 25 µL of different dilutions of the extracts (PE, EtOAc and *n*-BuOH) or standards (BHT, BHA and ascorbic acid) were added to 975 µL of DPPH (0.025 mg/mL). Then, the mixture was kept in the dark place at room temperature for 30 min. The absorbance was measured at 517 nm and the percentage of DPPH radical-scavenging activity of each extract was calculated as follows:

$$\text{DPPH scavenging effect (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

$A_{control}$ is the absorbance of blank and A_{Sample} is the absorbance of positive control or sample.

ABTS scavenging activity

The spectrophotometric analysis of ABTS scavenging activity was done by the method of Re et al [16]. The $ABTS^{\cdot+}$ was produced by the reaction between 7 mM of ABTS prepared in H_2O and 2.45 mM of $K_2S_2O_8$, stored in the dark at room temperature for 12 h before use. The ABTS solution was diluted with ethanol to get an absorbance of 0.70 ± 0.20 at 734 nm. Then, 2 mL of the diluted ABTS solution were added to 1 mL of sample solution (extracts, BHT and BHA) at different concentrations (0.0156 - l mg/mL). After 30 min, absorbances were measured at 734 nm and the percentage of inhibition was calculated for each concentration relative to a control absorbance. The scavenging capacity of ABTS was estimated using the following equation:

$$\text{Scavenging effect (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

$A_{control}$ is the absorbance of control and A_{Sample} is the absorbance of positive control or sample.

Reducing power assay

The reducing power was determined according to the method of Oyaizu et al [17], 100 µL of sample solution at different concentrations were added to 0.5 mL of phosphate buffer (0.2 M; pH 6.6) and 0.5 mL of potassium ferricyanide (1 %). The mixture was incubated at 50 °C for 20 min. Then 0.5 mL of trichloroacetic acid (10 %) was added to the mixture which was centrifuged for 10 min at 3000 rpm. The supernatant (0.5 mL) was mixed with 0.5 mL of distilled water and 125 µL of $FeCl_3$ (1 %) freshly prepared. The absorbances were measured at 700 nm and the results were calculated as $A_{0.5}$ (µg/mL) which indicated the concentration corresponding to the absorbance at 0.50 nm. The reducing power of the different extracts was compared to those of BHA, BHT, ascorbic and tannic acids and α-tocopherol as standards.

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the method of Apak et al [18]**.** 50 µL of a solution of $CuCl_2$ (10 mM) were added to 50 µL of the neocuprine solution (7.5 mM) and 60 µL of NH_4Ac buffer solution (1 M, pH = 7.0). Different concentrations of extracts and standards were added to the initial mixture to make a final volume of 200 µL. The samples were shielded from light and the absorbance was measured at 450 nm after 1h. The results were calculated as $A_{0.5}$ (µg/mL) and the reduction capacity of the extracts was compared with those of α-tocopherol, BHA and BHT as standards.

Chelation of ferrous iron

The chelation of ferrous iron was estimated by the method of Le et al [12]**.** 500 µL of the samples (extracts and EDTA) were initially mixed with 100 µL of $FeCl_2$ (0.6 mM) and 900 µL of methanol. After 5 min, 100 µL of Ferrozine (5 mM) were added. The obtained solutions were agitated and incubated for 10 min at room temperature and the absorbance was measured at 560 nm. The results of ferrous iron chelation were transmitted as percentage of inhibition (%) according to the equation:

$$\text{Activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

$A_{Control}$ is the absorbance at 560 nm of the control and A_{sample} is the absorbance of positive control or sample.

Chelation of the metal ions

The chelation of metal ions was assessed according to the method of Decker et al [19]**.** 40 µL of the samples (extracts and EDTA) at different concentrations were added to 40 µL of $FeCl_2$ (0.2 mM) and 40 µL of ethanol. The reaction was initiated by the addition of 80 µL of ferene solution (0.5 mM). The mixture was agitated vigorously and incubated for 10 min at room temperature. The absorbance was measured at 562 nm and the results were given as a percentage of inhibition using the following equation:

$$\text{Activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

$A_{Control}$ is the absorbance at 562 nm of the control and A_{sample} is the absorbance of positive control or sample.

***In vitro* cytotoxicity**

The cytotoxicity of the crude extracts prepared from the species *Scabiosa stellata* was evaluated according to brine shrimp *lethality essay* described by Meyer *et al* (1982) [20]**.** Brine shrimp eggs were hatched in a shallow rectangular dish (150 mm × 5 mm) filled with sea water (38 g marine salts dissolved in 1L of distilled water). The dish was divited into two unequal compartments using a plastic divider. The shrimp eggs (50 mg) were sprinkled into the larger darkened compartment, while the smaller compartment was illuminated. After 48 hours the phototropic nauplii were collected using pastor pipette from the illuminated compartment. The extracts in various concentrations (0.5, 1, 2, 4, 8 mg/mL) were prepared in dimethyl sulfoxide (DMSO). 100 µL of each prepared solution were transferred into vial tubes containing 4.9 mL of sea water and ten larvae of brine shrimps (nauplii) to obtain final concentrations of 10, 20, 40, 80 µg/mL. The vials were maintained under illumination. After 24 hours the survived shrimps were counted, the total death and the percentage of mortality of each dose were determined.

Acetylcholinesterase Inhibitory Activity

The inhibition of AChE by crude extracts was evaluated using the method described by Ellman et al [21]. 750 µL of sodium phosphate buffer (100 mM , pH 8.0) were added to 50 µL of crude extracts (PE, AcOEt and *n*-BuOH) at different concentrations and 100 µL of AChE (5.32×10^{-3} U) solution prepared in phosphate buffer salin pH = 8. The mixture was incubated at 25 °C for 15 minutes then 50 µL of DTNB (5,5′-dithio-bis-2-nitrobenzoate) (0.5 mM) were added with 50 µL of acetylthiocholine iodide (0.71 mM). The absorbance of the mixture was read at 412 nm, after 5, 10, 15 and 20 min. Galantamine was used as a positive control. The percentage of inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = (E - S/E) \times 100$$

Where E is the activity of the enzyme without extract and S is the activity of the enzyme with the extract.

Antibacterial Activity

The antibacterial activity of the crude extracts (PE, AcOEt and *n*-BuOH) was estimated by the agar disk diffusion assay [22] against ten bacterial strains including four Gram-positive (*Staphylococcus aureus* ATCC 25923, *Staphylococcus albus*, *Enterococcus* sp, *Streptococcus* D) and six Gram-negative (*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 15442, *Acinetobacter baumannii*, *Proteus mirabilis*, *Salmonella* Typhimurium and *Enterobacter sakazaki).* The bacterial strains used were initially isolated from patient affected with infections, identified in the Laboratory of Microbiology University Hospital Center (Batna) by microscopic examination stain reaction according to their morphology, Gram character, colony aspect, oxygen requirement, physiological and biochemical characters and using serological methods and purified using the method of streaking the four quadrants in sterile conditions and at optimum temperatures according to the strain concerned for 24 h. One or several colonies from each pure culture were transferred into 5 mL of nutrient broth. The bacterial suspension was homogenized and incubated at 37 °C for 10-24 hours. After incubation, a reading of the optical density (OD) of 1 mL of inoculum was made by a spectrophotometer at 625 nm. Opacity must be

equivalent to 0.5 McFarland. A sample from each inoculum was used to inoculate Petri disks containing Mueller Hinton by swabbing technique. Wathman paper disks (6 mm) were impregnated with 10 µL of the extract solutions at different concentrations (1000, 500, 250, 125, 62.5 and 31.25 mg/mL and filed carefully on the surface of the inoculated agar with sterile forceps. The discs of the negative controls were impregnated with DMSO. The Petri dishes were incubated at 37 °C for 24 h. The tests were performed in triplicate (three boxes for each concentration of extract and for each strain). The results were expressed by the diameters of zones of inhibition around the discs produced.

In vitro anti-inflammatory activity

The anti-inflammatory activity of *S. stellata* extracts was achieved by bovine serum albumin denaturation method [23]. 500 µL of sample solutions or standard drug (Ibuprofen) at different concentrations (125, 250, 500 and 1000 µg/mL) were added to 500 µL of BSA solution (0.2%) prepared in Tris Buffer Saline (pH 6.6). A control tube contains a volume of 0.5 mL of BSA and 0.5 mL of ethanol was also prepared. The tested tubes were incubated at 37 °C for 10 min then heated at 72 °C for 5 min. After cooling, the absorbances of these solutions were read at 660 nm. Each experiment was performed in triplicate and the average absorbance was recorded. The percentage of inhibition of denaturation was determined as follows:

$$\text{Inhibition of denaturation (\%)} = ((\text{A Control} - \text{A sample}) / \text{A Control}) \times 100$$

AControl is the absorbance at 660 nm of the control and Asample is the absorbance of positive control or sample.

Statistical Analysis

The results were given as the means ± S.D (p <0.05) for three replicates for each sample. The $A_{0.5}$ (reducing power and CUPRAC assay) and IC_{50} (DPPH, ABTS, anticholinesterase inhibition activity), LC_{50} (brine shrimp lethality test) and EC_{50} (chelation of ferrous iron, chelation of the metal ions) values were calculated by linear regression analysis.

Results and Discussion

Phytochemical screening

The phytochemical screening of crude extracts (PE, EtOAc and n-BuOH) obtained from the plant *S. stellata* L. reveals the presence of several classes of secondary metabolites well-known to possess pharmacological proprieties including : steroids, flavonoids, saponins, tannins, alkaloids, quinons and carotenoids. All the detected compounds were previously isolated from species of the same genus [24-28]. Flavonoids, saponins, triterpenoids and sterols have been previously purified and identified from this species [29-31].

Total bioactive contents

Regarding the importance of phenolic compounds as bioactive products doted of biological activities, the quantification of phenolic, flavonoid and tannin contents of *S. stellata* crude extracts were assessed. And the results are summarized in Table 01.

Table 1. Total bioactive contents of *Scabiosa stellata* extracts

Extracts	Total phenolic content [a] (µg GAE/ mg of extract)	Total flavonoids content [b] (µg QE/ mg of extract)	Total Condensed tannins content [c] (µg EC/mg of extract)
PE	30.33 ± 0.15	13.83 ± 0.45	0.07 ± 0.02
EtOAc	117.66 ± 0.52	72.90 ± 0.76	32.05 ± 0.15
***n*-BuOH**	77.12 ± 0.29	57.83 ± 0.25	0.95 ± 0.015

[a]Total phenolic content was expressed as µg gallic acid equivalent/ mg of plant extract ; [b] Flavonoid content was expressed as µg quercetin equivalent / mg of plant extract ; [c]Total condensed tannins content was expressed as µg catechin equivalent/ mg of plant extract.

A difference in total bioactive contents depending on solvent polarities used for the extraction procedures were observed**.** The highest phenolic, flavonoid and tannin contents were found in the ethyl acetate extract, followed by *n*-butanolic and petroleum ether extracts respectively.

Previous research conducted on the same species [29] and on species of the same genus including : *S. tschiliensis* [32] and *S. arenaria* [33] indicated lower levels of polyphenol and flavonoid contents compared to the results of the present study.

The variation in total phenolic content between species of the same genus could be due to various intrinsic factors (genetic potential of the individual species for the biosynthesis of polyphenols) and extrinsic factors (environment, stage of maturation and storage period) [34].

Antioxidant Activities

The results of the antioxidant activity are presented in Table 2. All the crude extracts exhibit an interesting antioxidant activity at different systems including: DPPH and ABTS radicals scavenging activities, reducing power, CUPRAC assay, ferrous ions and metal chelating assay. This result was expected, since several studies reported the considerable antioxidant potential of plants belonging to the same genus [32-33].

Table 2. Antioxidant activities of the species *Scabiosa stellata*

	Antioxidant activities					
	DPPH assay[a]	**ABTS assay[a]**	**CUPRAC assay[a]**	**Reducing power[a]**	**chelation in ferrous iron assay [a]**	**chelation in metal chelate assay [a]**
Extract and standards	IC_{50} (µg/mL)	IC_{50} (µg/mL)	$A_{0.50}$ (µg/mL)	$A_{0.50}$ (µg/mL)	EC_{50} (mg/mL)	EC_{50} (mg/mL)
***n*-BuOH**	21.22 ± 0.30	24.99 ± 0.36	42.16 ± 3.06	12.13 ± 0.52	1.65 ± 0.01	145.35 ± 0.34
EtOAc	25.15 ± 0.68	14.00 ± 0.8	28.50 ± 1.24	6.54 ± 0.48	5.03 ± 0.07	> 200
PE	171.61 ± 0.12	64.10 ± 0.38	100.9 ± 8.06	> 50	ND	ND
BHA[b]	6.82 ± 0.49	1.81 ± 0.10	3.64 ± 0.19	8.41 ± 0.67	NT	NT
BHT[b]	22.32 ± 0.02	1.29 ± 0.30	9.62 ± 0.87	> 50	NT	NT
Tannic acid[b]	7.74 ± 0.19	1.01 ± 0.16	3.76 ± 0.73	4.57 ± 0.30	NT	NT
Ascorbic acid[b]	3.1 ± 0.002	1.74 ± 0.10	12.43 ± 0.09	9.01 ± 0.46	NT	NT
α-Tocopherol[b]	13.02 ± 0.17	7.59 ± 0.53	19.92 ± 1.46	34.93 ± 0.38	NT	NT
EDTA[b]	NT	NT	NT	NT	0.063 ± 0.01	8.57 ± 0.14

[a]Values expressed are means ± SD of three measurements ($p < 0.05$); [b]Reference compounds; NT : not tested; ND: not determined because the inhibition at highest screened concentration (200 µg /mL) was less than 50%.

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All the crude extracts (PE, EtOAc and *n*-BuOH) possessed radical scavenging properties in both DPPH and ABTS assays that varies in a dose-dependent manner. In DPPH free radical scavenging assay, the *n*-butanolic extract (21.22 ± 0.30 µg/mL) exhibited a higher antioxidant activity then BHT (22.32 ± 0.02 µg/mL), but this activity was relatively low compared to the BHA (6.82 ± 0.49 µg/mL), ascorbic acid (3.1 ± 0.002 µg/mL) and α-tocopherol (13.02 ± 0.17) used as standards. In ABTS radical scavenging test, the EtOAc extract had strong antioxidant activity with a value of IC_{50} at 14.00 ± 0.8 µg/mL, followed by the *n*-butanolic and PE extract respectively.

In order to relate the antioxidant potential of the species *Scabisa stellata* to the presence of bioactive compounds that serve as electrons donor inducing the reduction of transition metals, the antioxidant activity was assessed by CUPRAC and reducing power assays (Table 2). The results of all the tested samples (extracts and standards) in both tests were affected in a dose-dependent manner. The EtOAc extract was the most potent extract, this

extract had high reducing power better than that of ascorbic acid, α-tocopherol and BHA known as common standards.

The results of the ferrous ion-chelating and chelation of the metal ions tests (Table 2) revealed that *n*-BuOH and EtOAc extracts had moderate metal chelating activities compared to EDTA as a positive control.While, PE extract does not possess any chelation activities at all the tested concentrations.

The interesting antioxidant activities of crude extracts at different systems can be attributed to the phenolic and flavonoids contents. In fact, several studies noticed that phenolic compound constitute the most powerful antioxidant agents isolated from plants [35].

In vitro cytotoxicity

All the extracts obtained from the plant *Scabiosa stellata* were subjected to Brine Shrimp lethality bioassay in order to evaluate their possible cytotoxic action and anti-tumor properties. The results are given in Table 3.

The result indicates that no mortality was observed with the control group treated with DMSO, while a concentration-dependent increment in a mortality rate of the brine shrimp nauplii was observed in groups treated with the *Scabiosa stellata* crude extracts. The *n*-butanolic extract was the most toxic to Brine Shrimp nauplii, with 57.2 ± 0.2 % of mortality at 80 µg/mL followed by the ethyl acetate extract (28.5 % of mortality) and the petroleum ether extract (42.8 % of mortality) at the same concentration (80 µg/mL).

Table 3. Result of *in vitro* cytotoxicity by Brine shrimp lethality bioassay

sample	**% Mortality under the studied concentrations (µg/mL)** [a]					**Toxicity Profile**
	100	**80**	**40**	**20**	**10**	
DMSO	**0**	**0**	**0**	**0**	0	Non-toxic
***n*- BuOH**	70.14±0.22	57.14±0.32	42.86±0.1	39.29±0.47	21.34±0.57	Toxic
EtOAc	32.02±0.92	28,57± 0.2	21,43± 0.26	17,86±0.3	14,29±0.3	Non-toxic
PE	47.2±0.83	42.86± 0.5	32.14± 0.3	21.43± 0.57	17.86± 0.2	Non-toxic

[a]Values expressed are means ± SD of three measurements ($p < 0.05$).

The significant cytotoxicity of the *n*-butanolic extract could be related to the chemical profile of the species *Scabiosa stellata*. Indeed, saponins isolated previously from this plant are known as potential antiproliferative and antitumor agents doted of apoptic and cytotoxic effects [36].

In vitro anti-inflammatory activity

The anti-inflammatory activity of *S. stellata* extracts was achieved by bovine serum albumin denaturation method and the results are presented in Table 4.

Table 4. In-vitro anti-inflammatory activity of *S. stellata* extracts

	Concentration (µg/ml)		
Extracts/standard	1000	500	250
PE	16 ± 0.1[a]	5.6 ± 0.1	4.0 ± 0.1
EtOAc	78.8 ±0.1	69.5 ±0.1	58.9 ±0.1
n-BuOH	3.2 ± 0.3	1.8 ± 0.1	-
Iboprofen[b]	100 ± 0.3	92 ±0.2	61 ± 0.2

[a]Values expressed are means ± SD of three measurements ($p < 0.05$); [b]Reference compounds; (-): not determined

All the crude extracts and the standard drug (Ibuprofen) had the ability to inhibit thermally-induced protein denaturation in a dose-dependent manner. Ibuprofen showed the maximum percentage of inhibition (100%) at the concentration of 1000 PPM. For the extracts, the maximum percentage of inhibition was observed in EtOAc (78.86%) extract followed by PE (16.02%) and *n*-BuOH (3.21%) extracts at the same concentration.

This result indicated the ability of various extracts obtained from the species *Scabiosa stellata* to prevent the alteration of the electrostatic, hydrogen, hydrophobic and disulfide bonds leading to maintain the three-dimensional structure of the proteins, and their capability to control the production of self-antigen in case of rheumatoid arthritis disease . The interesting anti-inflammatory activity of EtOAc extract can be attributed to the presence of phenolic and flavonoid compounds. Known to possess interesting biological properties [37].

Acetylcholinesterase inhibitory activity

The crude extracts of *S. stellata* were tested for their AChE inhibitory activities by spectrophotometric Ellman method . results showed that The ethyl acetate extract has a moderate activity (30.8%) at the concentration of 200 µg/mL, the *n*-BuOH extract has a low AChE inhibitory activity (10.9%) at the same concentration. While the PE extract did not show any AChE activity at all the tested concentrations.

Previous research conducted on crude extracts of the species *S. arenaria* [33] showed strong inhibition of AChE compared to the results of the present study, this variation in AChE activities among the species of the same genus could be explained by the difference in their chemical composition especially in polyphenols and flavonoids known for their anticholinesterase activities [38].

The results of the antibacterial activity are presented in Tables **5** . All the tested extracts showed inhibition of bacterial growth against at least two strains. The strains *Staphylococcus albus*, *Pseudomonas aregionosa* (ATCC 15442) and *Salmonella* Typhimurium are the most resistant strains to all plant extracts.

Table 5. Antibacterial activity of crude extractrs prepared from the species *Scabiosa stellata*

			Extracts		
	Bacterial strains	**Concentration (mg/ml)**	**PE**	**EtOAc**	***n*-BuOH**
Gram-negative strains	*Escherichia coli ATCC 35218*	1	12 ± 0.1[a]	10± 0.1	-[b]
		0.5	10 ±0.2	09± 0.2	-
		0.25	09 ± 0.2	-	-
		0.125	08± 0.1	-	-
		0.0625	-	-	-
	Pseudomonas aregionosa ATCC 15442	1	-	-	-
		0.5	-	-	-
		0.25	-	-	-
		0.125	-	-	-
		0.0625	-	-	-
	Acinetobacter baumannii	1	-	14 ± 0.1	10± 0.1
		0.5	-	10 ± 0.3	09 ± 0.1
		0.25	-	09 ± 0.1	08± 0.5
		0.125	-	-	-
		0.0625	-	-	-
	Proteus mirabilis	1	-	20± 0.1	-
		0.5	-	18± 0.4	-
		0.25	-	17± 0.1	-
		0.125	-	17± 0.3	-
		0.0625	-	16 ± 0.2	-
	Salmonella Typhimurium	1	-	-	-
		0.5	-	-	-
		0.25	-	-	-
		0.125	-	-	-
		0.0625	-	-	-
	Enterobacter sakazaki	1	-	-	10± 0.1
		0.5	-	-	09 ± 0.1
		0.25	-	-	08± 0.1
		0.125	-	-	-
		0.0625	-	-	-
Gram-positive strains	*Staphylococcus aureus ATCC 25923*	1	18 ± 0.2	13 ± 0.2	-
		0.5	15 ± 0.1	10 ± 0.1	-
		0.25	12 ± 0.3	09 ± 0.1	-
		0.125	09 ± 0.1	-	-
		0.0625	-	-	-
	Staphylococcus albus	1	-	-	-
		0.5	-	-	-
		0.25	-	-	-
		0.125	-	-	-
		0.0625	-	-	-
	Enterococcus sp	1	09± 0.1	09± 0.3	-
		0.5	-	-	-

	0.25	-	-	-
	0.125	-	-	-
	0.0625	-	-	-
Streptococcus D	1	-	09 ± 0.1	-
	0.5	-	-	-
	0.25	-	-	-
	0.125	-	-	-
	0.0625	-	-	-

[a]Values expressed are means ± SD of three measurements ($p < 0.05$) as the diameter of the zones of inhibition (mm) . [b] (-) no zones of inhibition around the discs.

The ethyl acetate extract was found to be the most effective extract against six strains .The greatest zone of inhibition was observed against the clinical strain *P. mirabilis* which is considered as a common cause of nosocomial and urinary infections and one of the most resistant strains [39].

The *n*-BuOH extract has an antibacterial activity against two Gram-negative bacteria *A. baumannii* and *E. sakazaki*. While the petroleum ether extract exhibited an inhibitory effect against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 35218).

The antibacterial activity observed in the tested extracts could be due to different classes of secondary metabolites purified and identified previously including flavonoids, saponins, triterpenoids and sterols, known for their antimicrobial activities [40].

Conclusion

The present study reported the pharmacological evaluation of the species *Scabiosa stellata*. The phytochemical screening of crude extracts indicated the presence of various types of secondary metabolites with important biological activities. important antioxidant activities in all the tested methods were observed and all the crude extracts inhibit thermally-induced protein denaturation in a dose-dependent manner and showed antibacterial and anticholinesterase activities.

Furthermore, it can be concluded that *S. stellata* extracts could be used as a good source of alternative natural products helpful in preventing oxidative and neurodegenerative diseases and as a source of antibacterial, anti-inflammarory and antiproliferative compounds.

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Do the Female Sea Turtles Benefit from Multiple Paternity? A Review of the Frequencies of Multiple Paternity Across Sea Turtle Rookeries

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Abstract: Sea turtles are promiscuous breeders. Since it is very difficult to observe individuals of a marine species while mating and usually impossible to determine the successful mating, molecular studies provide a tool to make an inference about mating system of this species. Recent molecular studies on sea turtle mating systems have demonstrated that polyandry is much more common than polygyny in sea turtles. It is well known that multiple paternity (MP) is evident in all sea turtle populations with polyandrous mating system. Determination of frequency of MP is of great importance for understanding of mating system and population structure of endangered populations and contributes to the conservation efforts. The frequency of MP shows great inter- and intra-specific variability. But why does this frequency vary greatly within and among species? Why does a female sea turtle mate multiple times within a season? Do the females benefit from MP? To elucidate these questions, here I review the frequency of MP for sea turtles nesting around the world. Based on data for several rookeries throughout the world, there were significant differences in the frequency of MP among species ($p < 0.01$). The frequency of MP was statistically correlated to neither clutch size (eggs) nor female size (curved carapace length [CCL]) ($p > 0.05$). However, there was a moderate positive correlation between the frequency of MP and hatching success (defined as the rate of hatchlings emerging successfully from the eggs) ($r^2 = 0.45$, $p < 0.05$). These findings suggest that MP, contrary to common belief, does not work in favour of larger females and does not result in increased clutch size, but hatching success increases with the increasing frequency of MP. It can be concluded from these evaluations that MP in sea turtle may have at least some benefits: increased genetic diversity and heightened offspring viability and variability.

Keywords: Sea turtle, Multiple paternity, Female size, Clutch size, Hatching success

Introduction

Mating System of a Species

Determination of a species' mating system is a crucial component of understanding natural history of that species (Bjorndal et al., 1983). Mating system is particularly substantial within small populations, since it may influence genetically effective size of population and evolution of the species (Arden & Kapuscinski, 2002; Charlesworth, 2009). Accurately estimating population size, population structure, and reproductive behaviour is of great importance to improve current conservation priorities and make effective management decisions on endangered species. In populations whose mating system is polyandrous, multiple paternity influences the effective population size (Sugg & Chesser, 1994) and the genetic variability within the population (Baer & Schmid-Hempel, 1999). Small population size and a skewed ratio of males to females available for mating at a nesting season may decrease genetic variation and adaptation ability to new environmental changes (Montgomery et al., 2000).

Sea Turtles

There are seven species of sea turtles living in the oceans: green turtle, *Chelonia mydas* (Linnaeus, 1758); loggerhead, *Caretta caretta* (Linnaeus, 1758); leatherback, *Dermochelys coriacea* (Vandelli, 1761); Kemp's ridley, *Lepidochelys kempii* (Garman, 1880); olive ridley, *Lepidochelys olivacea* (Eschscholtz, 1829); hawksbill,

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Eretmochelys imbricata (Linnaeus, 1766); and flatback, *Natator depressus* (Garman, 1880). They have been living for more than 100 million years (Hirayama, 1998), but they are under protection all around the world (Hamann et al., 2010). Today, sea turtles face numerous threats in each of their life stages (Spotila et al., 2000), which are caused by both natural and anthropogenic factors. Some of the natural factors influencing sea turtles are climate change, predation by domestic animals, erosive waves, and flooding (Wetterer & Lombard, 2010; Witt et al., 2010). Since sea turtles possess temperature-dependent sex determination (Bull, 1980; Wibbels et al., 2000), climate change is a unique threat to them; increases in temperature can result in extreme sex ratio biases (i.e., Mitchell & Janzen 2010). On the other hand, egg harvesting and turtle hunting (Kamezaki & Matsui, 1997), marine captures in fishery regions (Peckham et al. 2007), habitat degradation by coastal buildings (Kamezaki et al., 2003), and various types of pollution (Lewison & Crowder, 2007) are included to anthropogenic factors. Interactions of these and this kind of threats have caused a dramatic decline in sea turtle populations worldwide (Wyneken et al., 1988; Scherer-Lorenzen & Coomes, 2014), and that is why sea turtles are under protection. However, long-term monitoring and conservation projects have recently started to give the results in terms of population sizes (see Casale, 2015; Casale & Tucker, 2015).

Multiple Paternity in Sea Turtles

Sea turtles are promiscuous breeders, and both males and females may mate with multiple mates (FitzSimmons, 1998; Hamann et al., 2003). However, male sea turtles do not emerge on the beach and are difficult to observe at sea, the number of males contributing to a population is difficult to characterize. In addition, it is very difficult to observe individuals of sea turtles during the mating and generally impossible to determine the successful mating. Therefore, molecular studies provide an informative tool to make an inference about mating strategies of sea turtles. Recent molecular studies on sea turtle mating systems have demonstrated that in sea turtles, polyandry, which is described as mating of females with multiple males, is much more common than polygyny, which is described as mating of males with multiple females.

In sea turtle species, multiple paternity studies have been carried out over last several decades (i.e., Jensen et al., 2006; Theissinger et al., 2009; Figgener et al., 2016). These studies provide precious information regarding mating patterns and enable the researchers to understand population structure. The frequency of multiple mating is critical for understanding of the evolution of the mating systems and for the conservation of endangered populations (Kichler et al., 1999; Moore & Ball, 2002). It is well known that multiple paternity is evident in all polyandrous sea turtle populations (Sari et al., 2017).

Variations in the Frequency of Multiple Paternity Among Sea Turtle Species and Rookeries

It is believed that the frequency of multiple paternity shows great inter- and intra-specific variability. But why does this frequency vary greatly among and within species? To be able to answer this question, I conducted a literature search and reviewed the studies estimating the frequency of multiple paternity in sea turtle rookeries (described as breeding sites). Following this search, summary of the obtained data belonging to several multiple paternity studies on sea turtle species and rookeries were obtained (Table 1).

Table 1. Summary data of several multiple paternity studies on sea turtle species

Locality	Clutches analysed	Nesting females	Frequency of multiple paternity (%)	Marker	Study year	Reference
Green turtle, *Chelonia mydas*						
Ascension Island	18	18	61.0	Micro	1999-2000	Lee & Hays, 2004
Ascension Island	3	3	100.0	Micro	1999	Ireland et al., 2003
Tortuguero, Costa Rica	12	-	92.0	Micro	2007	Alfaro-Núñez et al., 2015
Kosgoda, Sri Lanka	24	19	47.0	Micro	2005-2006	Ekanayake et al., 2013
Melbourne Beach, Florida, U.S.A.	28	28	85.7	Micro	2011-2012	Long, 2013
Southern Great Barrier Reef, Australia	22	13	9.1	Micro	1991-1993	Fitzsimmons, 1998
Michoacán, Mexico	16	10	75.0	Micro	1998-2000	Chassin-Noria et al., 2017
Alagadi Beach, Cyprus	94	78	24.4	Micro	2008-2010	Wright et al., 2012
Cousine Island, Seychelles	9	3	0.0	Micro	2007-2008	Phillips et al., 2017
Alagadi Beach, Cyprus	94	78	24.4	Micro	2008-2010	Wright et al., 2013
Akyatan Beach, Turkey	22	22	59.0	Micro	2009	Turkozan et al., 2019
Loggerhead, *Caretta caretta*						
Zakynthos, Greece	20	15	93.3	Micro	2003-2004	Zbinden et al., 2007
Dalyan Beach, Turkey	25	10	70.0	Micro	2014	Sari et al., 2017
Wassaw Island, Georgia, U.S.A.	72	72	75.0	Micro	2008-2010	Lasala, 2011; Lasala et al., 2013
Mon Repos Beach, Queensland, Australia	29	29	65.5	Micro	2011-2012	Howe et al., 2017

Mon Repos Beach, Queensland, Australia	24	45	33.0	Allo	1982-1983	Harry & Briscoe, 1988
Melbourne Beach, Florida, U.S.A.	3	3	33.0	Micro	1994	Bollmer et al., 1999
Melbourne Beach, Florida, U.S.A.	70	70	31.4	Micro	1996	Moore & Ball, 2002
Dirk Hartog, Australia	14	NA	35.7	Micro	2013	Tedeschi et al., 2015
Bungelup, Australia	4	NA	25.0	Micro	2013	Tedeschi et al., 2015
Gnaraloo, Australia	7	NA	85.7	Micro	2011	Tedeschi et al., 2015
Gulf of Mexico, Florida, U.S.A.	51	51	70.0	Micro	2013-2015	Lasala et al., 2018
The Port of Nagoya Public Aquarium, Japan*	7	4	42.9	Micro	2000-2002	Sakaoka et al., 2011
The Port of Nagoya Public Aquarium, Japan*	11	4	27.3	Micro	2001-2003	Sakaoka et al., 2013
Hawksbill, *Eretmochelys imbricata*						
Bahía de Jiquilisco, El Salvador	41	34	11.8	Micro	2015	Gaos et al., 2018
Gulisaan, Malaysia	12	10	20.0	Micro	2004	Joseph & Shaw, 2011
Cousine Island, Seychelles	85	43	9.3	Micro	2007-2008	Phillips et al., 2013
Xicalango-Victoria, Campeche, Mexico	2	2	0.0	Micro	2011	González-Garza et al., 2015
Chenkan, Campeche, Mexico	16	10	0.0	Micro	2011	González-Garza et al., 2015
Celestun, Yucatan, Mexico	9	9	11.1	Micro	2011	González-Garza et al., 2015
El Cuyo, Yucatan, Mexico	4	4	0.0	Micro	2011	González-Garza et al., 2015
Las Coloradas, Yucatan, Mexico	12	10	8.3	Micro	2011	González-Garza et al., 2015
Holbox, Quintana Roo, Mexico	7	6	14.3	Micro	2011	González-Garza et al., 2015
Leatherback, *Dermochelys coriacea*						
Playa Grande, Costa Rica	50	20	10.0	Micro	1998-1999	Crim et al., 2002
Playa Gandoca, Costa Rica	35	18	22.2	Micro	2008	Figgener et al., 2016
St. Croix, U.S. Virgin Islands	38	12	41.7	Micro	2009	Stewart & Dutton, 2011
St. Croix, U.S. Virgin Islands	55	55	23.6	Micro	2010	Stewart & Dutton, 2014
Olive ridley, *Lepidochelys olivacea*						
Suriname	10	10	20.0	Micro	1995	Hoekert et al., 2002
Playa Hermosa, Costa Rica	13	13	30.8	Micro	2003	Jensen et al., 2006
Ostional, Costa Rica	13	13	92.3	Micro	2003	Jensen et al., 2006
Honduras	8	8	75.0	Micro	2012-2013	Duran et al., 2015
Kemp's ridley, *Lepidochelys kempii*						
Tamaulipas, Mexico	35	26	57.7	Micro	NA	Kichler et al., 1999
Flatback, *Natator depressus*						
Mon Repos Beach, Queensland, Australia	16	9	68.8	Micro	2004-2005	Theissinger et al., 2009

Micro refers to microsatellites and allo refers to allozymes.
* indicates that the study was carried out on captive sea turtles.
NA indicates missing data.

The frequency of multiple paternity varied greatly from 0% up to 100% among rookeries (Figure 1). After analysing these data, it was found that there were marked and significant differences in the frequency of multiple paternity among species (ANOVA, $F_{6, 36} = 4.06$, $p < 0.01$). For instance, hawksbill turtles had a significantly lower frequency of multiple paternity (8.3%) than both loggerhead turtles (52.9%) (t-test, $T = 6.27$, $df = 14$, $p > 0.001$) and green turtles (52.5%) (t-test, $T = 4.16$, $df = 11$, $p > 0.01$). Similarly, leatherback turtles had a significantly lower frequency of multiple paternity (24.4%) than both loggerhead turtles (t-test, $T = 3.04$, $df = 9$, $p > 0.05$) and green turtles (t-test, $T = 2.30$, $df = 12$, $p > 0.05$). Whereas the frequency of multiple paternity was uniform at hawksbill and leatherback turtle rookeries, it was more variable in other species. The great inter- and intra-specific variation in the frequency of multiple paternity detected in this study may be resulted from the differences in incidence of male-female encounters, population sizes, ratios of males to females available for mating at a nesting season, or breeding grounds or be considered as a consequence of the combination of these factors.

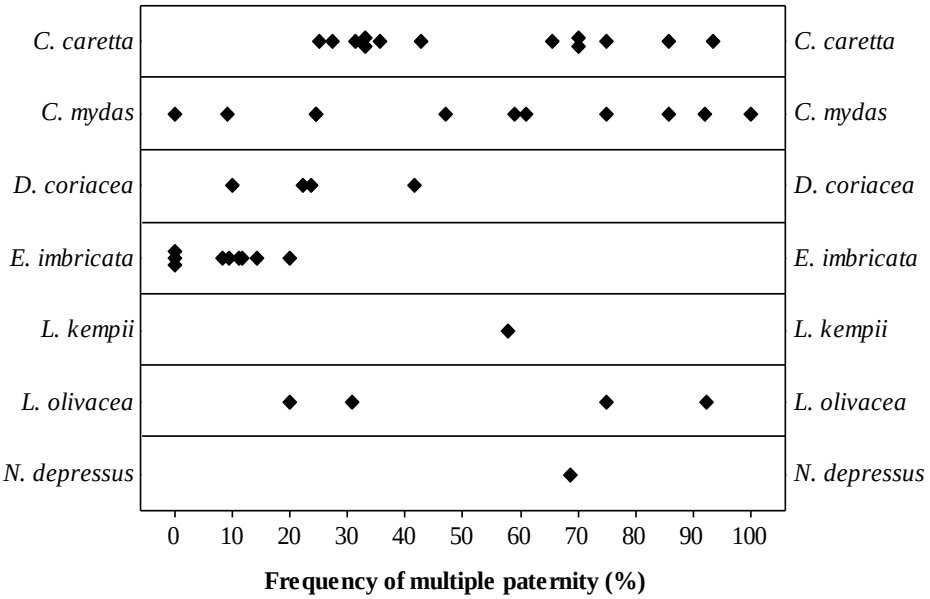

Figure 1. Variation in the frequency of multiple paternity in sea turtles.

Female Size vs Frequency of Multiple Paternity in Sea Turtles

Does the size of female sea turtles affect the frequency of multiple paternity? Is there a relationship between them? To explore this relationship (if there is), the results of the studies on multiple paternity involving female size data (curved carapace length [CCL]) (Table 2) were statistically analysed. It was found that the frequency of multiple paternity was not correlated to female size (Pearson correlation, $p > 0.05$). This finding implies that male sea turtles do not prefer the larger and hence older females, and larger female sea turtles do not prefer to mate with multiple males and are not acceptive for more than one male.

Table 2. Mean values of assessed parameters for sea turtle species from reviewed multiple paternity studies

Species	Frequency of multiple paternity (%)	Female size (cm)	Clutch size (eggs)	Hatching success (%)	Reference
Chelonia mydas	61.0	114.6	117.8	82.0	Lee & Hays, 2004
	92.0	108.7	117.6	85.8	Alfaro-Núñez et al., 2015
	47.0	106.5	NA	NA	Ekanayake et al., 2013
	85.7	107.2	130.0	66.7	Long, 2013
	24.4	NA	109.8	NA	Wright et al., 2012
	0.0	109.7	90.8	62.9	Phillips et al., 2017
	24.0	NA	111.4	NA	Wright et al., 2013
Caretta caretta	93.3	84.6	121.2	79.1	Zbinden et al., 2007
	70.0	76.9	82.6	87.0	Sari et al., 2017
	75.0	98.6*	114.7	78.3	Lasala, 2011; Lasala et al., 2013
	65.5	95.9	126.4	NA	Howe et al., 2017
Lepidochelys olivacea	30.8	NA	100.8	52.1	Jensen et al., 2006
	92.3	NA	99.5	74.6	Jensen et al., 2006
	20.0	NA	117.9	62.6	Hoekert et al., 2002
Eretmochelys imbricata	11.8	NA	173.4	NA	Gaos et al., 2018
Natator depressus	68.8	NA	55.0	83.0	Theissinger et al., 2009

* indicates that the female size value is straight carapace length (SCL), while the remaining are curved carapace length (CCL).
NA indicates missing data.

Clutch Size vs Frequency of Multiple Paternity in Sea Turtles

It is believed that larger female sea turtles have larger pelvic opening structures compared with those of smaller ones, and this structure constrains egg size and hence offspring size. Since larger females can accumulate more resources and/or bigger eggs, because of their larger pelvic opening, they can therefore produce more eggs (Wilbur & Morin, 1988). Therefore, the relationship between clutch size and frequency of multiple paternity reported by the reviewed studies (Table 2) was statistically analysed. Accordingly, no statistical correlation was found between them (Pearson correlation, $p > 0.05$).

Frequency of Multiple Paternity vs Hatching Success in Sea Turtles

Why does a female sea turtle mate multiple times within a season? Do the females benefit from multiple paternity? Increased offspring viability, offspring genetic diversity, fertilisation assurance, and procurement of compatible gametes are believed to be some of the benefits of multiple paternity (FitzSimmons, 1998; Uller & Olsson, 2008). It has been assumed by Sari et al. (2017) that one of the simplest ways to investigate viability of the offspring is to investigate hatching success (defined as the rate of hatchlings emerging successfully from the eggs). To see the relationship between frequency of multiple paternity and hatching success (if there is), data reported by the studies for these two parameters (Table 2) were statistically analysed. Accordingly, a moderate positive correlation between the frequency of multiple paternity and hatching success was detected (Pearson correlation, $r^2 = 0.45$, $p < 0.05$) (Figure 2). This finding suggests that multiple paternity results in increased hatching success and multiple paternity contributes to the persistence of the populations, since the hatchlings which are able to emerge from the eggs and then from the nests are strong enough to crawl on the sand and to swim in the ocean.

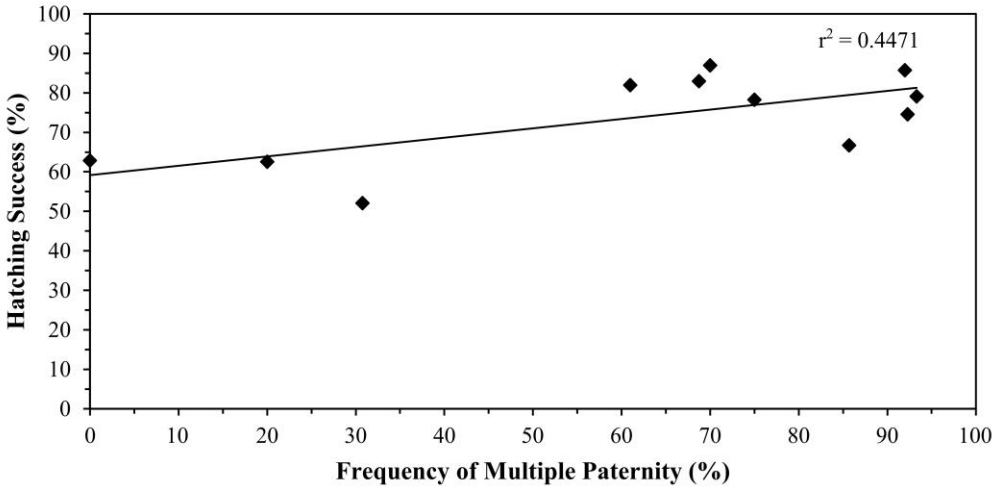

Figure 2. Relationship between frequency of multiple paternity and hatching success in sea turtles.

Conclusion

The frequency of multiple paternity varies greatly from 0% up to 100% among rookeries, and it shows marked and significant differences among species. It can be suggested that multiple paternity, contrary to common belief, does not work in favour of larger females and does not result in increased clutch size, but hatching success increases with the increasing frequency of multiple paternity. It can be concluded from these evaluations that multiple paternity in sea turtle may have at least some benefits: increased genetic diversity and heightened offspring viability and variability.

Recommendations

Multiple paternity studies have great importance, since they provide valuable and crucial information about reproductive behaviour of sea turtles. Multiple paternity levels of sea turtle populations should be taken into account for management and conservation strategies, since they influence effective population size and

diversity. Genetic diversity plays a key role in the ability of the sea turtle species to adapt themselves to environmental alterations and their survival in the future.

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Evaluation of Drinking Water Quality in Pogradec District

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Abstract: Supplying quality safe drinking water is a key factor for human health, a fundamental human right and an important component of effective health protection policies. In some regions of our country, investments in water supply and sanitation are lacking, affecting people's health. In this study, we have determined the quality of drinking water in Pogradec district, through organoleptic testing, physicochemical properties and bacteriological indicators, total coliforms and *Escherichia coli*. In this study are analyzed water samples, during each season of 2018, taken from Progrdec city and village piped system, in a natural recourse and private wells, which people use for water supply. Analyzes have been compared to drinking water rates according to the World Water Organization (WHO). The data obtained shows that the water supply in the city is clean and recommended for drinking and other personal needs. Organoleptic parameters are normal, physicochemical and bacteriological properties are in the limits recommended by WHO. Except in some cases where the parameters are not normal, there was a network fault, but immediate measures were taken to resolve the problem. In private wells, only one of the three was contaminated with total coliform and *E. coli*. This is because the well was not built under the right conditions. We recommend investing in the city and village water supply network and disinfecting them regularly. Also, private wells should be well maintained and disinfected. We also need to be careful with soil fertilization, because it affects groundwater pollution.

Keywords: Water supply, Well, Total coliforms, *Escherichia coli*

Introduction

Drinking water quality parameters are often the most important tools for measuring access to improved water sources. The drinking water with an acceptable quality shows the water safety in terms of its physical, chemical and bacteriological parameters (WHO, 2004). Some of the attributes, including color, turbidity, odor, hardness, etc, are substantially influenced by the acceptability of drinking water (Addisie, 2012). Having a good knowledge of the factors that influence public perception can help improve water management, consumer services, the acceptance of water reuse and risk communication, among other areas (Doria *et al.,* 2009). Therefore, consumer perceptions and aesthetic criteria need to be considered in assessment of drinking water supplies even if they may not adversely affect human health (WHO, 2004). Safety is increased if multiple barriers are in place, including protection of water resources, proper selection and operation of a series of treatment steps and management of distribution systems (piped or otherwise) to maintain and protect treated water quality. The great majority of evident water-related health problems are the result of microbial (bacteriological, viral, protozoan or other biological) contamination. Cases with infections caused by contact or consumption of contaminated water with pathogenic bacteria such as *Escherichia coli* have been reported from different parts of the world, often causing the epidemic leading to death (Angulo *et al*., 1997).

Monitoring for the presence of pathogenic bacteria is a fundamental issue of water quality assessment, where direct or indirect use leads to serious human health problems (Chapman, 1992). According to WHO (2004), about 80% of all diseases and over 1/3 of deaths in developing countries are caused by contaminated drinking water. According to the previous studies (Osmani *et al*., 2019) the situation of water supply infrastructure in Albania is in a critical state as a result of economic problems. In some regions of our country, investments in water supply and sanitation are lacking, affecting people's health. People, who have opened wells to satisfy their needs, didn't analyze the water quality. Accurate data on this process are not available; however, two international NGOs have done basic surveys in rural areas where piped systems were absent (PIA, 2001).

In this study, we have determined the quality of drinking water in Pogradec district, through organoleptic testing, physicochemical properties and bacteriological indicators, total coliforms and *Escherichia coli*. In this study are analyzed water samples, during each season of 2018, taken from Progrodec city and village piped system, in a natural recourse and private wells, which people use for water supply. Analyzes have been compared to drinking water rates according to the World Water Organization (WHO) 2008.

Method

Area of the Study

The city of Pogradec was built on the southwestern shores of Lake Ohrid and is one of the most notable cities of Albania for its tradition in hosting "family tourism" and for the pleasant, fresh climate during the summer season.

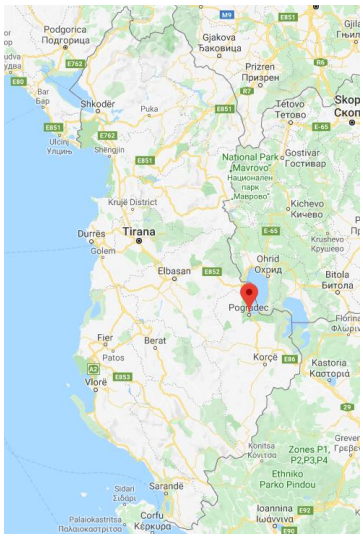

Figure 1. Map of Albania and the Pogradec city

The tectonic lake is four million years old and is the deepest of its kind in the Balkans**,** reaching a maximum depth of 285 meters. The lake environment is a natural habitat for a variety of old flora and fauna. It houses the rare fish "Koran," a kind of trout, impossible to find in almost any other lake in the world. Lake Ohrid is part of the Natural and Cultural Heritage List of UNESCO (Basler, 2000).

Sample Collection and Analysis

Water samples are taken in every season during 2018. In the Pogradec city are taken eight samples in the network and in the village are taken eleven samples; seven in network, one in natural recourse and three in private wells. In the table 1 are given the places where are collected the sample.

Table 1. The places of water samples in Pogradec city and village

No	Pogradec city	Village
1	Turizmi	Depozita
2	Kala	Lagje
3	Rruga Naim Frashëri	Cërravë
4	Spitali	Zërvaskë
5	Vërdova	Guras
6	Axhensia	Bucimas
7	1 Maji	Natural recourse
8	Dëshmorëve	Private wells 1
9		Private wells 2
10		Private wells 3

Water samples were taken according to WHO (1997). Water samples for bacteriological analysis were obtained via 250 ml sterile bottles, where the date and place of sampling was noted. In natural resources, water samples were taken holding the bottle by the lower part; submerge it to a depth of about 20 cm, with the mouth facing slightly upwards. While in wells and pipes, we have turn on the tap at maximum flow and let the water run for 1-2 minutes. While holding the cap and protective cover face downwards (to prevent entry of dust, which may contaminate the sample), immediately we have hold the bottle under the water jet, and fill.

(a) (b)

Figure 2. Water sample collection (a) piped system and (b) private well

The collected drinking water samples have been tested in the regional laboratory of Health directory in Pogradec.

Organoleptic parameters

Color. Transparency (visibility) of water is a measure of depth of penetration of light. This parameter depends on the presence of coloring matter and turbidity due to suspended matter. Color of water (hue) can be due to organic or inorganic contaminants. It can also be pH-dependent. Color of water, free from suspended matter, can be estimated semi-quantitatively by comparing samples with standard solutions of potassium chromate of different dilutions.

Odor and Taste. Odor and taste determinations are qualitative and subjective. In addition to chemical and biological effects of foul smelling and coloring constituents, they make the water aesthetically unacceptable. Odor in water is a general sign of pollution by decaying organic matter. Compounds that contribute to odor are generally volatile organic compounds, while chemicals that contribute to taste and odor are ketones, phenols, aldehydes and some other organic and inorganic compounds (Bitton, 2005).

Physicochemical properties

pH is dertermined according to BS EN ISO 10523:2012, with electrode. The potential of the measuring electrode is a function of the hydrogen ion activity of the measuring solution.
Chlorine (Cl) The quickest and simplest method for testing for chlorine residual is the DPD (diethyl paraphenylene diamine) indicator test, using a comparator. A tablet of DPD is added to a sample of water, colouring it red. The strength of colour is measured against standard colours on a chart to determine the chlorine concentration. The stronger the colour, the higher the concentration of chlorine in the water.
Nitrite (NO_2^-) is determined according to EN 26777:2006 by spectrophotometer.
Amonia (NH_3) is determined according to ISO 7150/1:1984 by spectrophotometer with Nessler reagent (mixing K_2HgI_4, NaOH or KOH).

Bacteriological properties

The membrane filtration method, ISO 9308-1 (2002) was used for the detection and enumeration of *Escherichia coli* and total coliform bacteria in water for human consumption.

Results and Discussion

Organoleptic Parameters

The standards that establish water's quality criteria for human consumption include organoleptic analysis. Organoleptic analysis of water are related to odor, taste and color. If the water has an unusual taste or smell (or it is cloudy or colored), it can be interpreted as a health risk and a problem in the water source, its treatment, or in the water network. Transparency of water is related to coloring materials and turbidity due to suspended and colloidal matter. Certain inorganic as well as organic compounds change the organoleptic properties of water or even make it unfit for human or industrial uses.

Table 2. Organoleptic properties in water sample from Pogradec city

No	Water sample	Spring			Summer			Autumn			Winter		
		Color	Odor	Taste	Color	Odor	Taste	Color	Odor	Taste	Color	Odor	Taste
1	Turizmi	N	N	N	N	N	N	N	N	N	N	N	N
2	Kala	N	N	N	N	N	N	N	N	N	N	N	N
3	Rruga Naim Frashëri	N	N	N	N	N	N	N	N	N	N	N	N
4	Spitali	N	N	N	N	N	N	N	N	N	N	N	N
5	Vërdova	N	N	N	No	No	N	N	N	N	N	N	N
6	Axhensia	N	N	N	N	N	N	N	N	N	N	N	N
7	1 Maji	N	N	N	N	N	N	N	N	N	N	N	N
8	Dëshmorët	N	N	N	N	N	N	N	N	N	N	N	N
9	WHO, 2008	N	N	N	N	N	N	N	N	N	N	N	N

N-Normal

No- No normal

According to WHO (2008) pure (normal) drinking water, it should have no color, odor and taste. In all water samples in the city the organoleptic characteristics are normal, with the exception of the sample taken in Vërdova. Where during the summer season abnormal values in appearance and odorles have occurred. According to the residents supplied by this water network, they found that the water was not translucent (it was brown) and had odorles. Then, they informed the local government who had noticed a pipeline rupture. Immediately measures were taken to regulate it and the water returned to normal.

In the village water supply, except for the samples taken in Zërvaskë and Guras, the values are according to WHO (2008). In these two places, as a result of the amortization of the water pipes, the pipe was damage. This has caused the odor of water. Measures have been taken by the local government to solve the problem.

Table 3. Organoleptic properties in water sample from village

No	Water sample	Spring			Summer			Autumn			Winter		
		Color	Odor	Taste	Color	Odor	Taste	Color	Odor	Taste	Color	Odor	Taste
1	Depozita	N	N	N	N	N	N	N	N	N	N	N	N
2	Lagje	N	N	N	N	N	N	N	N	N	N	N	N
3	Cërravë	N	N	N	N	N	N	N	N	N	N	N	N
4	Zërvaskë	N	No	N	N	N	N	N	N	N	N	N	N
5	Guras	N	No	N	N	N	N	N	N	N	N	N	N
6	Bucimas	N	N	N	N	N	N	N	N	N	N	N	N
7	Natural recourse	N	N	N	N	N	N	N	N	N	N	N	N
8	Private wells 1	N	N	N	No	No	No	N	N	N	N	N	N
9	Private wells 2	N	N	N	N	N	N	N	N	N	N	N	N
10	Private wells 3	N	N	N	N	N	N	N	N	N	N	N	N
9	WHO, 2008	N	N	N	N	N	N	N	N	N	N	N	N

N-Normal
No-No normal

In Table 3, from the analysis of the three private wells, it was found that only one, during the summer season, had color, odor and taste. The water of this well was contaminated. Soil fertilization with compost and livestock compost, decreased rainfall and soil irrigation (as a result of high temperatures in summer) have affected the composition of the compounds along with the water in the well water. Also, this well was very old and was not built according to the standards of construction.

Physicochemical Properties

However, WHO (2008) recommends that public water systems maintain pH levels of between 8.2 and 8.8, a good guide for individual well owners. Water with a low pH can be acidic, naturally soft and corrosive. Acidic water can leach metals from pipes and fixtures, such as copper, lead and zinc. It can also damage metal pipes and cause aesthetic problems, such as a metallic or sour taste, laundry staining or blue-green stains in sinks and drains. Water with a low pH may contain metals in addition to the before-mentioned copper, lead and zinc. Drinking water with a pH level above 8.8 indicates that a high level of alkalinity minerals are present. High alkalinity does not pose a health risk, but can cause aesthetic problems, such as an alkali taste to the water that makes coffee taste bitter; scale build-up in plumbing; and lowered efficiency of electric water heaters. İn our study the pH was in the limits of WHO (2008).

Many of the diseases that affect traumatized communities are caused by micro-organisms carried in drinking-water. Hence the reference to water-borne diseases. Disinfection is the process of destroying these organisms to prevent infection. There are a number of methods of disinfecting water, but chlorination is by far the most common. According to WHO in 2008 Guidelines, based on taste considerations, a guideline value of 5 mg/l was established for chlorine. Chloramination may give rise to the formation of nitrite within the distribution system, and the concentration of nitrite may increase as the water moves towards the extremities of the system (WHO, 2011). İn our study chlorine was below the limit in the city figure 3 and village samples figure 4.

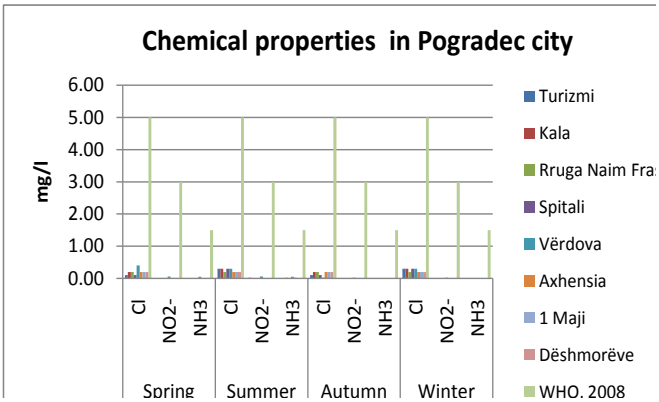

Figure 3. Chemical properties in water sample from Pogradec city

Nitrites are part of the nitrogen cycle, that is the transformations of nitrogen and nitrogen containing compounds in nature. While nitrites alone do not signify a pollution problem, their presence in combination with ammonia and nitrate may indicate environmental contamination. Nitrites can enter water through the use of corrosion inhibitors in industrial process water, or through the conversion from ammonia or nitrates. Nitrite can also be formed chemically in distribution pipes by *Nitrosomonas* bacteria during stagnation of nitrate-containing and oxygen-poor drinking-water in galvanized steel pipes or if chloramination is used to provide a residual disinfectant and the process is not sufficiently well controlled (WHO, 2011). In our study nitrites are under the limits of WHO (2008) 3 mg/l.

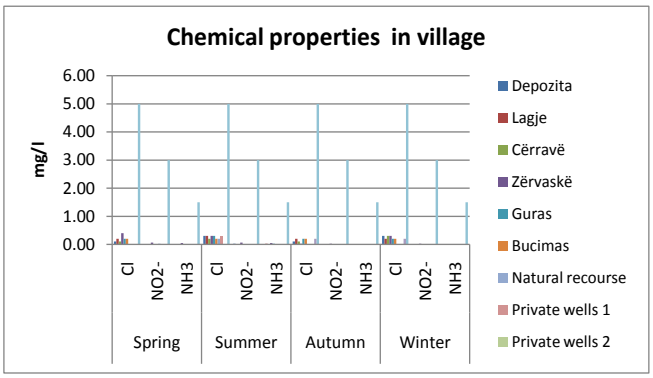

Figure 4. Chemical properties in water sample from village

Natural levels in groundwaters are usually below 0.2 mg of ammonia per litre. Higher natural contents (up to 3 mg/litre) are found in strata rich in humic substances or iron or in forests (Dieter & Möller, 1991). Ammonia may be present in drinking-water as a result of disinfection with chloramines. The presence of ammonia at higher than geogenic levels is an important indicator of faecal pollution (ISO, 1986). Taste and odour problems as well as decreased disinfection efficiency are to be expected if drinking-water containing more than 0.2 mg of ammonia per litre is chlorinated, as up to 68% of the chlorine may react with the ammonia and become unavailable for disinfection (Wendlandt, 1988). In our study the ammonia under the limit of WHO (2008) 1.5 mg/l.

Bacteriological Properties

Dirty and polluted water can contain many harmful organisms including pathogenic bacteria, which cause diseases like cholera, bacillary dysentery, typhoid, and diarrhea. Disinfection of water aims to kill these pathogens without leaving any harmful chemical substances in the water. Coliform bacteria, thermotolerant (faecal) coliforms and Escherichia coli have for almost a century been used as indicators of the bacterial safety of drinking water (Osmani *et al*., 2019). Water quality guidelines state that drinking water must not contain waterborne pathogens. More specifically, *Escherichia coli* or total coliforms should not be present in any 100 ml sample of drinking water (WHO, 2008).

Table 3. Bacteriological properties in water sample from Pogradec city

No	Water sample	Spring		Summer		Autumn		Winter	
		Total coliform	*E. coli*	Total coliform	*E. coli*	Total coliform	*E. coli*	Total coliform	*E. coli*
1	Turizmi	0	0	0	0	0	0	0	0
2	Kala	0	0	0	0	0	0	0	0
3	Rruga Naim Frashëri	0	0	0	0	0	0	0	0
4	Spitali	0	0	0	0	0	0	0	0
5	Vërdova	0	0	0	0	0	0	0	0
6	Axhensia	0	0	0	0	0	0	0	0
7	1 Maji	0	0	0	0	0	0	0	0
8	Dëshmorët	0	0	0	0	0	0	0	0
9	WHO, 2008	0 / 100 ml							

The water in city is not contaminated with pathogenic bacteria, because all the water saples have 0 total coliform/100 ml water and 0 E. coli/100 ml water . This as a result of regular disinfection and the investments made in network. İt is recommended for drinking water and other needs.

Table 3. Bacteriological properties in water sample from village

No	Water sample	Spring		Summer		Autumn		Winter	
		Total coliform	*E. coli*	Total coliform	*E. coli*	Total coliform	*E. coli*	Total coliform	*E. coli*
1	Depozita	0	0	0	0	0	0	0	0
2	Lagje	0	0	0	0	0	0	0	0
3	Cërravë	0	0	0	0	0	0	0	0
4	Zërvaskë	0	0	0	0	0	0	0	0
5	Guras	0	0	0	0	0	0	0	0
6	Bucimas	0	0	0	0	0	0	0	0
7	Natural recourse	0	0	0	0	0	0	0	0
8	Private wells 1	0	10	0	2	0	0	0	0
9	Private wells 2	0	0	0	0	0	0	0	0
10	Private wells 3	0	0	0	0	0	0	0	0
11	WHO, 2008	0 /100ml							

Also in the village network, the water samples are not contaminated with pathogenic bacteria. Only the first private well is contaminated, because it has > 0 *E. coli*/ 100 ml water. So it is contaminated with *E. coli*. This pollution has come from over-utilization of livestock compost, fertilizers, pesticides etc., to increase the soil fertility. Inappropriate ways of irrigation and rainfall have affected the penetration of coliforms in the soil depths and underground water.

Conclusion

Based on the data obtained, we came to the conclusion that the water supply in the city is clean and recommended for drinking and other personal needs. Organo-leptic parameters are normal, both physico-chemical and bacteriological are within the limits recommended by WHO (2008). Except in some cases where the parameters are not normal, there was a network demage, but immediate measures were taken to resolve the problem.

Even in the village's water supply and natural recourse water is clean and recommended for drinking and personal use. Where the parameters studied are within the limits recommended by the WHO. In one of the private well, was contaminated with *Escherichia coli.* This is because the well was not built properly and was not insulated. This pollution has come from over-utilization of livestock compost, fertilizers, pesticides etc., to increase the soil fertility. Inappropriate ways of irrigation and rainfall have affected the penetration of coliforms in the soil depths and underground water.

Recommendations

Presently, rural water supply systems remain in desperate need of improvement, even though many of them are well beyond cost-effective repairs and interventions that are more painstaking should be considered. Should be paid attention to the investments in the development of the village’s water supply with pipes (24 hours/day), investments in the sanitary sewer and their disinfection should be done regularly. The wells must fulfill the

hygienic sanitary norms. Also, disproportionate use of livestock, fertilizers, pesticides etc., should be avoided. People need to be informed about the importance of ideal water supply and the impacts of water polluted have on their health.

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Investigating the Effects of Fertigation on the Clogging and Uniformity Distribution of Emitters

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Abstract: Emitter clogging is one of the drip irrigation systems' main challenges, which the water quality plays a crucial role in this regard. So, in the current study, a suitable physical model for implementing fertigation in drip irrigation system was established. The hydraulic performances of three types of emitters (labeled type 1 to 3) having discharge rates of 4 L/hr were evaluated. For this purpose, the amounts of manufacturing variation coefficient, pressure-discharge relation, application uniformity and Christiansen's Uniformity Coefficient (UC) were determined. Additionally, some experiments were conducted for evaluating the emitter clogging in fertigation with different urea concentrations of 0, 50, 150 and 250 g/m^3, during 480 hrs. It was revealed that the emitter type 3 had superior performances in comparison with two other emitters in most cases. Moreover, according to the obtained results, the percentage of emitter clogging was increased by increasing the time duration of the experiment and the concentration of urea. As a conclusion, the emitter type 3 was recommended for implementing fertigation in drip irrigation systems due to its high distribution uniformity and more resistance against clogging in comparison to others.

Keywords: Drip irrigation, Emitter clogging, Fertigation, Hydraulic performance

Introduction

The water crisis is one of the essential issues in arid and semi-arid regions such as Iran. This condition has gotten worse in recent years due to occurring successive droughts. Considering the indices of the water crisis, Iran's water resources have a very critical condition. Therefore, proper management of water allocation is vital in these resources. Moreover, the section of agriculture is the primary water consumer in Iran. In another point of view, trickle irrigation is one of the irrigation methods which its high abilities in water saving and labor cost reduction have been mentioned in the literature.

Different factors such as emitter clogging, manufacturing variation and temperature and pressure fluctuations affect the emitter discharge and distribution uniformity and may decrease the efficiency of the system (Keller and Bliesner, 1990). So, emitter clogging is one of the main problems for the optimum utility of drip irrigation systems in the orchards and fields. Emitter clogging causes non-uniform distribution of water in the fields and decreases the crop yield (Dehghanisanij et al., 2005).

Bozkurt and Ozekici (2006) studied different effects of the fertigation on emitter clogging and concluded that fertilizers containing calcium and phosphate led to sever clogging of emitters. Nakayama and Bucks (1981) categorized emitter clogging by the physical, chemical and biological factors. Haijun and Guanhua (2009) investigated the performance of three types of long-pass, on-line and pressure compensating emitters using fresh

water and treated sewage effluent. Results indicated that the water quality, emitter type and irrigation duration influenced the emitter clogging and distribution uniformity. Bo Zhou et al. (2015) examined drip irrigation emitter clogging using reclaimed water and then the effect of different irrigation frequencies on dynamic emitter's outflow was studied. The results indicated that emitter clogging increased with shorter drip irrigation intervals.

The objective of the current research is to evaluate the resistance of three types of emitters against clogging in fertigation with different concentrations (0, 50, 150 and 250 gr/m^3) of Urea by the nominal emmission rate of 4 L/hr.

Methods

A physical model was constructed to investigate the performance and hydraulic characteristics of the emitters including the manufacturing coefficient of variation, pressure-discharge relation, distribution uniformity and emitter clogging by applying different levels of Urea concentration. The mentioned model was consisted of a 150L reservoir tank and 48 emitters as depicted in Figure 1.

Figure 1. Constructed physical model

Three types of common emitters in Iran were selected for experiments (Figure 2). The apparatus was operated under the pressure of 10 m-H_2O and then the discharges of emitters were measured.

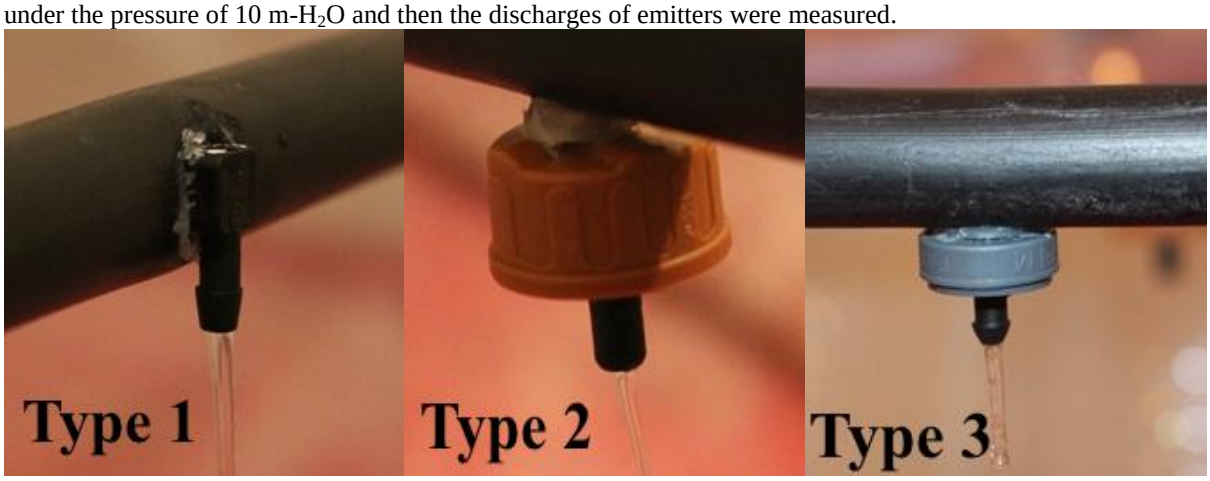


Figure 2. Three types of utilized emitters in the experiment.

The experiment was implemented with four concentrations of Urea (0, 50, 150 and 250 gr/m^3). Water temperature during the experiment was almost constant at 20 ˚C. Furthermore, the system was operated 8 hours per day during 60 days of experiment. The average discharge of each emitter during its application was determined at the end of every day.

Gene Expression Programming (GEP)

Gene expression programming (GEP) is an algorithm that utilizes populations of individuals and chooses them based on their fitness, and it can apply genetic changes by the usage of genetic operators (Ferreira, 2001a, b). The first stage in the GEP algorithm concerns creating a primary population of solutions. Next, the chromosomes represented as a tree expression, which assessed according to a fitting function. According to the selection, the best individuals have more chances of having children. The whole process is repeated for some generations, and as the new generations appear, it is expected that population quality improves on average.

Evaluation parameters

The performances of GEP models were evaluated by Correlation Coefficient (CC), Root Mean Square Error (RMSE), Willmott's Index of agreement (WI) and Mean Absolute Error (MAE). These statistics are presented as follows:

$$CC = \frac{\left(\sum_{i=1}^{n} O_i P_i - \frac{1}{n}\sum_{i=1}^{n} O_i \sum_{i=1}^{n} P_i\right)}{\left(\sum_{i=1}^{n} O_i^{\,2} - \frac{1}{n}\left(\sum_{i=1}^{n} O_i\right)^2\right)\left(\sum_{i=1}^{n} P_i^{\,2} - \frac{1}{n}\left(\sum_{i=1}^{n} P_i\right)^2\right)} \quad (1)$$

$$WI = \left|1 - \left[\frac{\sum_{i=1}^{N}(O_i - P_i)^2}{\sum_{i=1}^{N}\left(\left|P_i - \bar{O}_i\right| + \left|O_i - \bar{O}_i\right|\right)^2}\right]\right|, 0 \le WI \le 1 \quad (2)$$

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(P_i - O_i)^2} \quad (3)$$

$$MAE = \frac{1}{n}\sum_{i=1}^{n}|P_i - O_i| \quad (4)$$

where O_i and P_i are observed and predicted ith value.

Results and Discussion

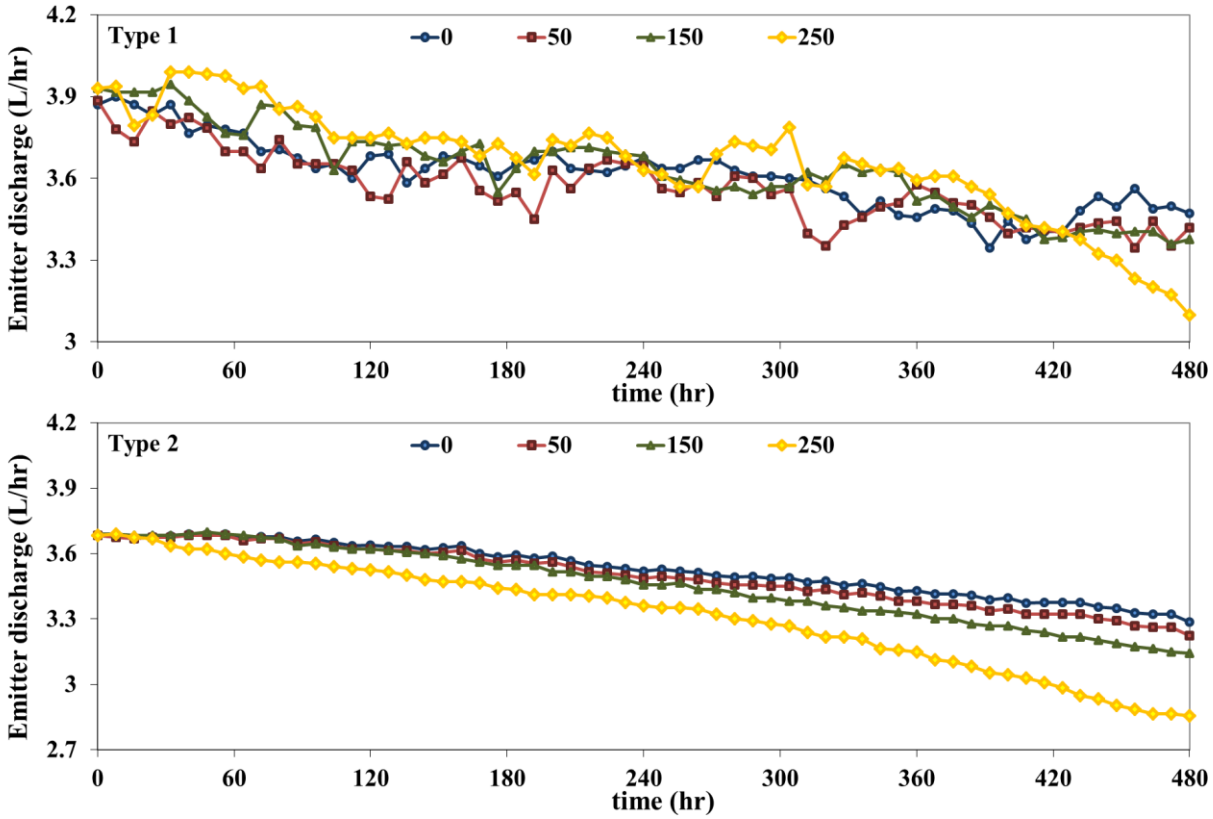
The hydraulic charachtersitics of the three emitter types were determined in different pressures during the experiments as presented in Table 1. The nominal discharge of the emitters was 4 L/hr, but real measured discharges measured during the experiments were a little different. Although the distribution uniformity of the emitter type 2 was a little higher than emitter type 3, emitter type 3 may be recommended because of its lower differences from nominal discharge of 4L/hr. Meanwhile, it can be comprehended from Table 1 that discharge of emitter type 3 was less sensitive to the pressure variations and had an acceptable performance comparing to other studied emitters.

Table 1. Hydraulic characteristics of utilized emitters

Hydraulic characteristic	Emitter type	Pressure (m-H_2O)		
		8	10	12
Average discharge (L/hr)	1	3.82	3.90	4.14
	2	3.82	3.68	3.75
	3	4.14	4.04	4.10
manufacturing variation coefficient	1	0.041	0.074	0.035
	2	0.025	0.024	0.024
	3	0.024	0.032	0.027

Christiansen's Uniformity	1	96.50	95.19	97.10
	2	97.94	97.97	97.95
	3	98.00	97.44	97.63
distribution uniformity	1	94.99	91.15	95.65
	2	97.15	97.25	97.40
	3	96.73	96.47	96.80
Discharge variation	1	-4.5	-2.5	3.5
	2	-4.5	-8.0	-6.3
	3	3.5	1.0	2.5

In the second stage of the experiment, the clogging of the emitters was investigated by applying four different concentration of 0, 50, 150 and 250 g/m^3 Urea during 480 hrs and the obtained results are illustrated in Figure 3. According to the results, the discharges of all emitters were decreased during 60 days of experiment. The performance of emitter type 3 was better than other emitters. As can be seen from Figure 3, emitter type 3 has lower discharge decreasing trend in comparison with other types.



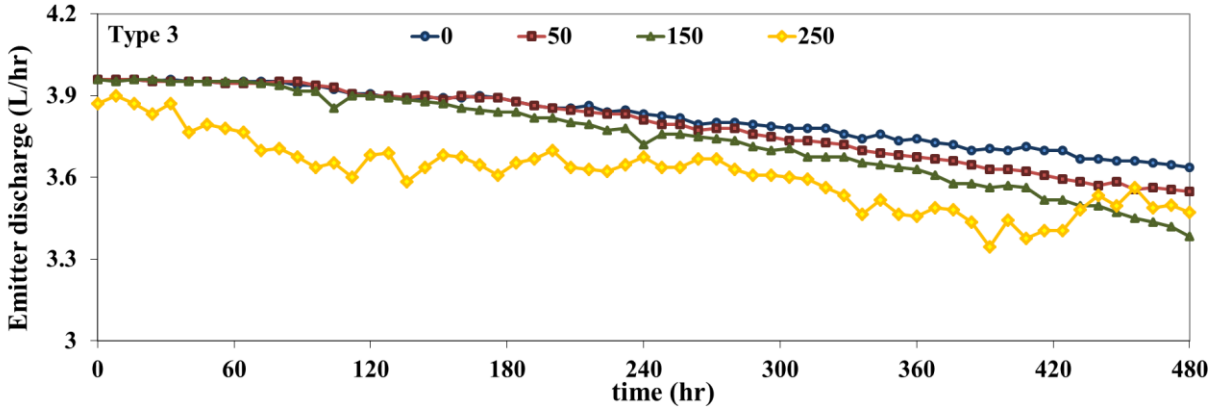


Figure 3. The comparison of emitter discharges under different concentrations of Urea fertigation.

For extracting mathematical formulations for each emitter, the discharge rates of them were collected and sorted randomly. Then, 70% and 30% of data were used for the calibration and validation process of GEP models, respectively. The obtained statistical results for the three studied emitters are presented in Table 2.

Table 2. Statistical parameters for each emitter

Emitter type	Statistical parameters			
	RMSE	MAE	CC	WI
1	0.116	0.087	0.726	0.838
2	0.047	0.038	0.969	0.984
3	0.088	0.05	0.864	0.992

It can be comprehended from Table 2 that the predictions of emitter type 2 are more accurate than other emitters. Additionally, the resulted formulations of the GEP model for the emitter types 1 to 3 are presented with equations 5 to 8, respectively.

$$Q = ArcTan[\frac{1}{\sqrt{(1+(4.939+0.100384T)^2)}}]^{1/6} + ArcTan[\frac{1}{\sqrt{(1+(6.17999+0.119143T)^2)}}]^{1/6} + ArcTan[(-17.2247+2C-T)^2]^2 \quad (5)$$

$$Q = 3.25949 + 0.581383\cos[0.185307T^{1/3}] - 0.0010082\,6(-9.97263-T)\times\sin[9.2905-C]^3 \quad (6)$$

$$Q = 3.20105 - \frac{\sin[5.88669-T^{1/3}]}{8.46362-\sqrt{C}+C} + \sin[\sin[e^{\sin[T^{1/4}]^3}]] - \tan[0.006224\tan[0.003112T]] \quad (7)$$

where, Q is the emitter discharge (L/hr), T is time (hr) and C is the Urea concentration (g/m^3).

Overall results indicated that the emitter type 3 showed the best performance against clogging. Therefore, it may be a right choice for the application of fertigation by drip irrigation systems.

Conclusion

In the current research, three emitter types were examined for their resistance against clogging. For that purpose, four different concentrations of Urea including 0, 50, 150 and 250 g/m^3 were injected during 480 hrs to the system and temporal discharge rates of emitters were measured. The obtained results indicated that the clogging rates were increased during 60 days of the experiment. Moreover, the emitter type 3 showed the best performance and was selected as the superior emitter type.

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The Effect of Egg Yolk Oil on the Mast Cell Concentration in Excisional Wound Healing of STZ-Diabetic Rats

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Abstract: Diabetes mellitus (DM) is serious metabolic disease. Impaired wound healing in DM leads to significant morbidity and mortality due to various clinical and socioeconomic issues. Wound healing is a complex mechanism involving different tissues and cells. Mast cells (MC) are the first group of cells to respond to injury and contribute to three main phases of wound healing: inflammation, proliferation, and scar formation/remodeling. The aim of this study was to evaluate the effect of egg yolk oil (EYO) on MC concentration in excisional wounds of streptozotocin (STZ) diabetic rats. Female Sprague-Dawley rats were allocated in three groups (6 rats per group) as: Group 1 (non-diabetic, topically treated with 2% fusidic acid ointment), Group 2 (STZ-diabetic, topically treated with 2% fusidic acid ointment), and Group 3 (STZ-diabetic, topically treated with EYO). On third day after single intraperitoneal injection of 65 mg/kg STZ, two full-thickness skin excisional wounds were generated on the back of all rats (day 0). On day 7 and day 14, randomly selected three rats per group were sacrificed under deep anesthesia. Skin sections were stained with toluidine blue, and MC numbers were determined. Differences in these numbers among the groups were analyzed statistically. Group 2 and Group 3 had statistically higher MC concentration on day 0 compared to Group 1 (p<0.001). Group 3 had statistically lower MC concentration on day 7 compared to Group 1 and Group 2 (p<0.01). In addition, increased MC degranulation was observed in Group 3 on the same day. The results of this study suggest that EYO induces MC degranulation, which is related to wound healing process, and decreases MC concentration in the first few days of the wound healing in STZ-diabetic rats. This decrease in MC concentration in DM is likely to be enable the wound to heal earlier than some other cases.

Keywords: Wound healing, STZ, Egg yolk oil, Mast cells, Diabetes mellitus

Introduction

Wound healing is a complex and systematic progression, and consisting three phases: inflammation, proliferation, and remodeling (Falanga, 2005). It has been documented that unresolved inflammation causes chronic wounds (Nguyen et al., 2019). In diabetic patients, chronic foot ulcers caused by impaired wound healing are common, and morbidity, mortality, and excess care costs are quite high (Ramsey et al., 1999).

Mast cells (MC) are found abundantly in skin like barrier organs, and they initiate allergic reactions. It has been shown that MC enhance acute inflammation, stimulate re-epithelialization and angiogenesis, while they promote scarring in normal wound healing; their numbers increase in chronic wounds, hypertrophic scars and keloids (Gniadecki, Gajkowska, Bartosik, Hansen, & Wulf, 1998; Komi, Khomtchouk, & Santa Maria, 2019; Noli & Miolo, 2001). MC granules contain many mediators such as heparin, histamine, tryptase, chymase, vascular endothelial growth factor, and tumor necrosis factor alpha; they release these mediators by degranulation during wounding, accelerate the angiogenesis and fibroblast proliferation and increase collagen synthesis (Artuc, Hermes, Steckelings, Grützkau, & Henz, 1999; Hatamochi, Fujiwara, & Ueki, 1984; Tonnesen, Feng, & Clark, 2000). It has been also reported that MC accumulate at the wound edge and may participate the collagen remodeling (Iba, Shibata, Kato, & Masukawa, 2004). When all these are taken into account, it can be suggested that MC can be involved in three phases of wound healing, which are inflammation, proliferation and remodeling phases.

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In recent years, the importance of MC in chronic diseases and wounds has been recognized, and effects of many natural products on MC concentration have been investigated (Estevão et al., 2015; Farahpour, Hesaraki, Faraji, Zeinalpour, & Aghaei, 2017; Kempuraj, Caraffa, Ronconi, Lessiani, & Conti, 2016; Ozay et al., 2018; Sarandy et al., 2018; Shi & Shi, 2012; Souza Neto Junior et al., 2017). For example, Farahpour, Mirzakhani, Doostmohammadi, & Ebrahimzadeh (2015) have reported that, *Pistacia atlantica* hulls ointment enhances MC distribution and infiltration, which in turn promotes the neovascularization, and it can therefore be considered as an appropriate compound for wound healing medicine. This study aimed to evaluate the effect of egg yolk oil (EYO) on MC concentration in excisional wounds of streptozotocin (STZ) diabetic rats.

Method

Preparation of EYO

To obtain EYO, the method which was developed by Herring & Ala (1980) was used. Boiled fifteen organic chicken eggs' shells were removed, egg whites were separated, and only solid egg yolks were put in the cleaned and sterilized pan. After that, the egg yolks were heated up to 190-200 °C for 20 minutes to release EYO. When EYO come out from the egg yolk, it was stirred for another 5 minutes. Then, pure EYO was obtained by throwing redundant egg yolks and filtering it from sterile gauze. EYO was kept in an amber flask at +4 °C until further use in this study.

Experimental Animals and Study Design

All rats were fed commercial rat feed and water *ad libitum*, and were kept on a 12 h light/12 h dark cycle. Three groups of rats which were consisted randomly selected six female Sprague-Dawley rats (190-230 g) were allocated as: Group 1 (non-diabetic, topically treated with 2% fusidic acid ointment), Group 2 (STZ-diabetic, topically treated with 2% fusidic acid ointment), and Group 3 (STZ-diabetic, topically treated with EYO). On third day after single intraperitoneal injection of 65 mg/kg STZ (dissolved in 0.1 mol/L sodium citrate buffer, pH 4.2), the rats with fasting blood glucose level of 250 mg/dL and above were considered as diabetic. All ethical approvals were taken from the Pamukkale University Animal Experiments Ethics Committee (PAUHDEK 2015/3).

Excision Wound Model

Excision wounds were created according to Nagappan, Segaran, Wahid, Ramasamy, & Vairappan, 2012. The dorsal thoracic skin of the anesthetized rats was shaved with electric razor, hairs were removed and the region disinfected using 70% alcohol (Figure 1A). Two mutually rounded wounds were formed on the back of each rat (Figure 1B and C) using sterile punch-biopsy apparatus (a diameter of 6 mm). For this purpose, the punch-biopsy apparatus applied to the shaved area with a uniformly pressure single twist until the subcutaneous dermal layer was separated (day 0). The wounds were cleaned using sterile gauze and compressed. All rats were put into cages separately. Before the applications of ointments, all wounds were cleaned with sterile saline solution. After that, all wounds were covered with sufficient ointments (Ozay et al., 2013) (Figure 1D and E). 2% fusidic acid was applied to Group 1 and Group 2, and EYO was applied to Group 3. Applications to the wound areas were continued for 14 days, once a day.

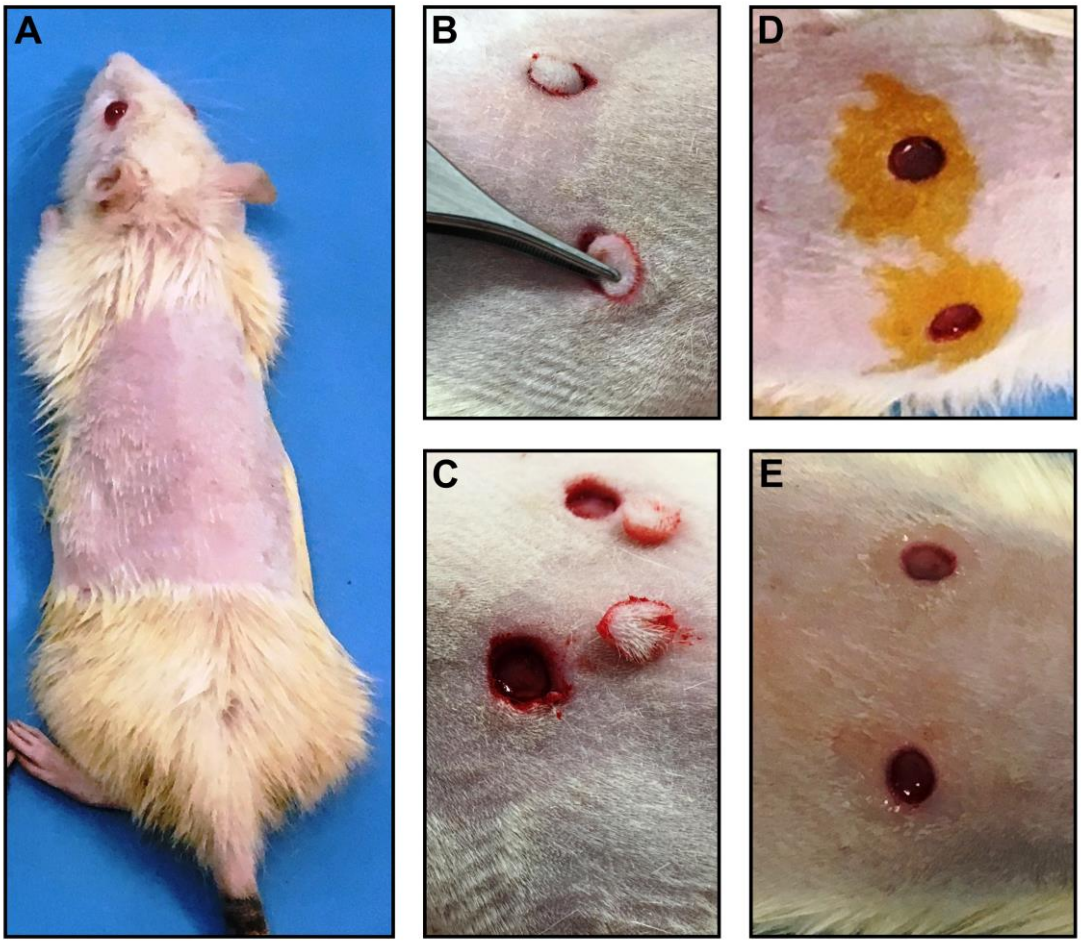

Figure 1. Excision wound model in the study

On day 7 and day 14, randomly selected three rats from each group were sacrificed under deep anesthesia. Wound skin samples were fixed in 10% formalin and embedded in paraffin after routine tissue processing. 5 µm skin sections were stained with toluidine blue (TB) (Toluidine Blue Staining Protocol for Mast Cells, n.d.) and analyzed with light microscope. MC numbers were determined by counting in the randomly selected five 400X fields by two observers.

Statistical Analyses

All values were expressed as means ± standard deviation (SD). The differences in the numbers of the MC among the groups were analyzed using the Kruskal-Wallis test and significant differences among groups were evaluated by the Mann-Whitney U test using Minitab 16 (Minitab Inc., State College, PA). All p values <0.05 were considered to be statistically significant.

Results and Discussion

MC were examined in the randomly selected microscopic fields (Figure 2) and counted for each sampled rat per examined day.

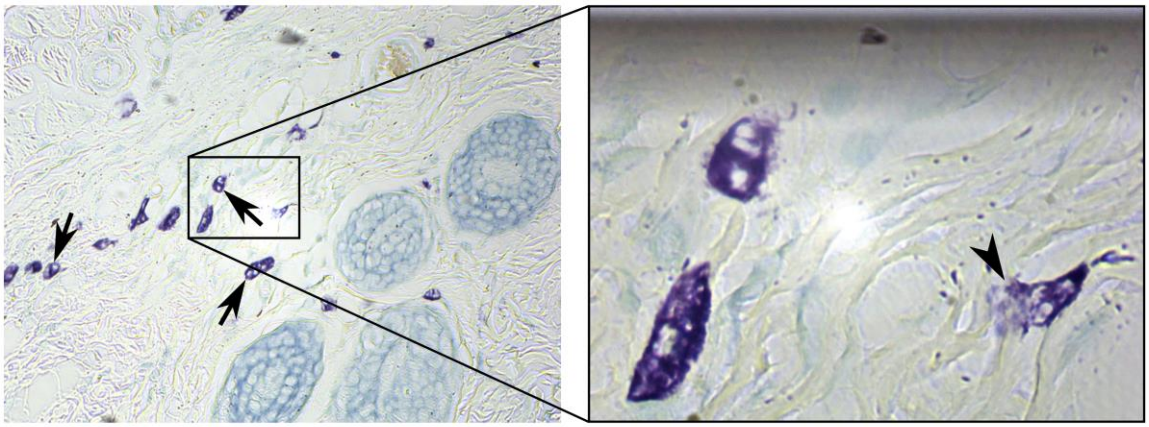

Figure 2. Photomicrographs of excisional skin wounds in rats. Note numerous MC (arrows) in the skin and the degranulation of MC in inset (arrowhead). Toluidine blue. ×200. Inset ×1000.

There were no significant differences among the groups in terms of MC concentration regardless of the days of lesion ($p>0.05$), but there were among the days of lesion regardless of the groups ($p<0.001$). Descriptive data on topical applications in the rats are presented in Table 1. In Group 1, MC concentration tended to decrease consistently, which was lower on day 7 compared with day 0 and lower on day 14 compared with day 7. However, in Group 2 and Group 3, MC concentrations decreased on day 7 compared with day 0, and then they showed an increase compared with day 7, but not more than the concentrations on day 0. Group 2 and Group 3 had statistically higher MC concentration (17.20 and 17.72, respectively) on day 0 compared to Group 1 (12.48) (Figure 3) ($p<0.001$). Group 3 had statistically lower MC concentration (4.87) on day 7 compared to Group 1 (10.93) and Group 2 (8.90) (Figure 3) ($p<0.01$). In addition, increased MC degranulation was observed in Group 3 compared with Group 1 and Group 2 on day 7. On day 14, there were no significant differences among the numbers of MC of the groups.

Table 1. Effects of EYO topical application on MC concentration in STZ-diabetic rats

Groups	**Day 0**		**Day 7**		**Day 14**	
	Mean	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**
Group 1	12.48[a]	0.86	10.93[a]	1.46	7.80[a]	0.66
Group 2	17.20[b]	2.19	8.90[a]	1.47	11.37[a]	2.11
Group 3	17.72[b]	1.65	4.87[b]	0.58	11.83[a]	2.26

Means that do not share a superscript letter within same column are significantly different from each other ($p<0.05$).

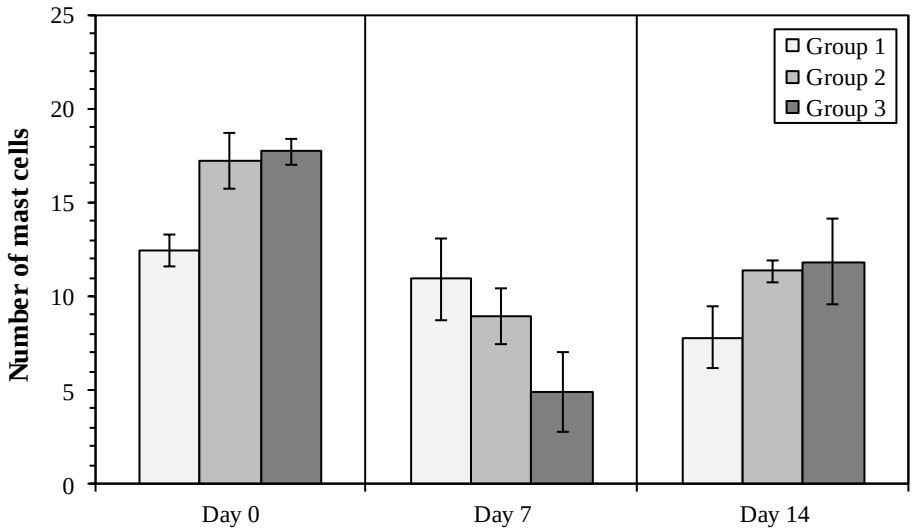

Figure 3. MC concentration of the groups in the study on days 0, 7, and 14.

In diabetic patients' skin, high numbers of MC have been observed in some studies (El Safoury, Fawzy, El Maadawa, & Mohamed, 2009; Tellechea et al., 2016). Similar to these studies, it was found in the present study that the DM groups (Group 2 and Group 3) had higher MC concentrations than Group 1 on day 0. This result may have been due to the chronic proinflammatory state on the skin caused by diabetes (Leal et al., 2015; Tellechea et al., 2013).

It has been reported that the number of MC significantly decreases in adelmidrol + trans-traumatic acid treated diabetic wounds (Siracusa et al., 2018). It was detected in the present study that EYO significantly reduced MC concentration in Group 3 on day 7 compared to Group 1 and Group 2. This result is in agreement with the results of the study carried out by Babaei et al. (2017) in which the effects of omega-3 fatty acids on diabetic wounds were investigated. It has been also suggested that the eggs of the poultry are one of the source of the omega-3 fatty acid (Shinn, Proctor, & Baum, 2018). Taking this knowledge into account, it can be stated that the omega-3 fatty acid content of the EYO may cause this reducing effect on MC concentration.

Activation of MC and release of histamine are necessary processes for normal wound healing (Weller, Foitzik, Paus, Syska, & Maurer, 2006). MC, by means of degranulation, also release many mediators, and these mediators are triggered and modulated inflammatory stage, connective cellular elements proliferation, and remodeling of connective tissue matrix (Noli & Miolo, 2001), which are the phases of wound healing process. It has been shown that MC degranulation increases in diabetic human and mice skin (Nishikori, Shiota, & Okunishi, 2014). In the present study, increased MC degranulation was mostly observed in Group 3 on day 7 compared to Group 1 and Group 2. In light of this finding, it can be assumed that EYO triggers the MC degranulation, and hence causes a decrease in MC concentration and consequently accelerates the wound healing by affecting epithelial cells and fibroblasts. These cells are involved in matrix formation and remodeling (Noli & Miolo, 2001). Similarly, it has been found by Souza Neto Junior et al. (2017) that 10% *Ximenia americana* containing ointment decreases MC concentration and accelerates the early wound contraction in rat skins. It has been also assumed by the same researchers that degranulated MC rapidly disappears and therefore MC cannot be identified in histological sections easily (Souza Neto Junior et al., 2017).

Conclusion

The results of this study suggest that EYO induces MC degranulation, which is related to wound healing process, and decreases MC concentration in the first few days of the wound healing in rats with DM. This decrease in MC concentration in DM is likely to be enable the wound to heal earlier than some other cases.

Recommendations

It can be recommended from the results of this study that EYO may be a promising therapeutic agent for the DM-caused wound healing, but further molecular analyses are needed to investigate the active compounds and the mechanisms of their actions.

Acknowledgements

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Life History Characteristic and Larval-Pupal Parasitoids of the Dolichandrone Weevil, *Cionus* sp. (Coleoptera:Curculionidae)

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Abstract: Mangrove trumpet trees (*Dolichandrone serrulata* (Wall. ex DC.) Seem are popular in Thailand and used as edible plants and ornamental trees. *Cionus* sp.(Dolichandrone weevil) feeds on Mangrove trumpet trees but data on its life history is very limited. Based on field collection from the Mangrove trumpet tree from public parks in Nonthaburi province, Thailand and laboratory studies, it provides the morphological description of larvae and adults, new data on growth and development of *Cionus* sp. as well as its larval-pupal parasitoids. The insect species has four different life stages: egg, larva, pupa and adult. After adult emergence for 5-7 days, mating will occur during daytime and the copulation lasts for 5-10 hours. The female laid eggs in a group of 3-7 eggs. A minimum of 14.71 days is required for passing through the egg, larval and pupal stages. Females live slightly longer than males (82.35 and 81.20 days, respectively). Upon the completion of larval development, it will build a cocoon, pupate in there and attach to the ventral or dorsal part of leaf surface. The adults normally emerge in the morning. It exibits death-feigning behaveior when being disturbed. In nature, both adults and larvae are commonly found in the rainy season. These insect larvae feed on leaves and young twig surface of Mangrove trumpet trees whereas adults prefer young leaves. Heavy infestation can stunt the tree's growth and cause dieback. The larvae and pupae were observed parasitized by the hymenopteran parasitoid, *Entedon* sp. The parasitization occurrence during May, 2017 to July, 2018 was 6.25-24.19% in larvae and 0.00-62.50 % in papa. Further studies should be conducted to evaluate the efficacy of *Entedon* sp. as the potential biological control agent for *Cionus* sp population reduction in home gardens and residential areas.

Keywords: Death-feigning behavior, *Entedon* sp., Growth and development, Lifespan, Natural control

Introduction

Mangrove Trumpet Tree (*Dolichandrone serrulata* (Wall. ex DC.) Seem is a small to medium-sized trees up to 10-20 meters and a species of plant in the Bignoniaceae family, order Lamiales. It is widely distributed in Bangladesh, Myanmar, Thailand, and Vietnam. It has a straight, robust and cylindrical trunk with a large, broadly conical and shady crown. Compound leaves are composed of 5-7 pairs of leaflets arranged opposite one another. White trumpet-shaped flowers are found in cluster 3-7 flowers(Veesommai and Kavduangtain, 2004). Fruits is flatten and has a characteristic flat like a bean pod which the pods are flat, parallel, curved, twisted, up to 1 m long These deciduous trees are found throughout all parts of Thailand. It is popular as boiled vegetable for local consumption and herbal drink. Currently, it is planted as ornamental plants because of the beautiful shrubs. In Thailand, there are 3 species of plants in a genus *Dolichandrone*: *D. columnaris*, *D. spathacea*, and *D. serrulata*. Regarding *D. serrulata*, are found in the forest, fields, mixed deciduous forest, flowering during March to June.

Insect pest of *D. serrulata* are the lesser death's head hawkmoth (*Acherentia styx*), dark bordered hawk moth (*Psilogramma increta*). Both of them are leaf feeders(Bangpai et al.,2017; Namee, 2017). Leaf beetle (*Alticini* sp.) and *Dolichandrone* weevil (*Cionus* sp.) also found attacked *D. serrulata*. Dolichandrone weevils are an

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important insect pest of the Mangrove trumpet tree. Cunningham (1979) described the development of *Cionus* sp. and indicated that the mortality of young instars was quite high. The larvae have muscous, hyaline covering to protect them from desiccation and against cannibalism. Upon fully development the larvae, chitinous strands secretion from the peritrophic membranes, and mixed with glutinous material of the hyaline coat flows around the larval body, covering the ventral surface. Then, it turns into an ovoid cocoon(Rather,1989). The larvae feed on the surface of young twigs near the young shoots whereas the adults are leaf feeders. Rather (1989) noted that during the development of different life stages of the *Cionus* weevil prefer and attack different parts of its host plant.

Entedon sp. is a parasitic wasp of beetles in the families Curculionidae, Brentidae, Anobiidae, Chrysomelidae, Buprestidae, Cerambycidae, Mordellidae, and Nitidulidae (Askew and Kopelke 1989, Gumovsky, 2006; Rasplus, 1991). *E. cionobius* is a parasitoid of *Cionus* spp. (Alford, 2019). The larvae and cocoons of *C. hortulanus* and *C. tuberculosis* are attacked by a gregarious, internal parasitoid, the eulophid *Entedon cionobius* (Thomson) (Rather,1989). The solitary, *Habrocytus cioni* (Thomson) is external pteromalid also common in the area and known as an ectoparasite of both species including *Gelis* spp. and two ichneumonids: *Itoplectis alternans* (Grav.) and *Agrothereutes abbreviator* (Fabr.). There were no parasitoids of the eggs or adults of Cionini species. However, *Entedon* sp. is an egg parasitoid of *Cirina forda* (Odebiyi et al., 2003).

Previous studies indicated that very few information on the Dolichandrone weevils is available. Therefore, the biology and morphology of this insect should be investigated to find best available methods for further management program. This research will focus on life history, morphology of all stages of insect growth and its parasitoids.

Methods

Morphological Characteristics of Dolichandrone Weevil

All life stages of Dolichandrone weevil was investigated under a stereomicroscope (Olypus SZX 10). Color, size and characteristic body of egg, larva, pupa and adult was recorded, measured and photographed.

Life History of Dolichandrone Weevil

The studies on general biology, host infestration and devolpment of *Cionus* both in the field and in the laboratory.

Field Experiment

Larvae, cocoon and adults of Dolichandrone weevil were collected monthly (May, 2017 and July, 2018) from public parks in Muang District of Nonthaburi, Thailand for life history. The difference of host infestation caused by both larvae and adults was evaluated.

Rearing in the Laboratory

Collected cocoons were placed in plastic boxes (14x19x6 cm). After adult emergence, the fresh leaves of *D. serrulata* is provided as food. The copulation time is recorded. Number of egg laid in a petiole cavity was counted. The growth and development of different life stages is investigated including coccoon building and related information.

Parasitization of Dolichandrone weevil by *Entedon* sp.

Larvae and pupa of Dolichandrone weevil were collected from public parks in Muang District of Nonthaburi from May, 2017 to July, 2018. These insects were kept in rearing containers under the laboratory condition (30 ^{o}C and 70%RH). Young leaves of *D. serrulata* is provided as food for the larvae until they underwent the pupation process. Whereas the collected coccoons were placed in a small plastic glass(1 cocoon/cup). Parasitization was investigated including sex ratio of male to female. Rate of parasitization was calculated in paercentage.

Results and Discussion

Morphological Characteristic of Dolichandrone weevil

Description of Immature Stage

The detailed of size dimension was described in Table 1.
Egg: round, oval, light yellow, 0.49 mm wide and 0.86 mm long
Larva: 3 immature stages, legless, slug-like larva**,** mucus secretion to cover body to prevent the body surface from drying out or natural enemy protection.
1st instar larva: small sized, body length 1.89 mm and 1.01 mm wide, brownish black head 0.30 mm wide, thorax and abdomen pale yellow and being dark color with mucous.
2nd instar larva: body 3.46 mm long and 1.77 mm wide, head width 0.51 mm, head slightly black color, black body with mucous cover**.**
3rd instar larva: body 6.62 mm long and 3.21 mm wide, head width 0.82 mm, without mucous body brownish yellow, head black or brown, fat and short body, legless, not clearly segmented, exuviae retaining in cocoon.
Pupa: Exerate pupa, 4.80 mm long and 3.37 mm wide**.**

Description of Male

Geniculate clubbed antennae with scape (first longest segment), funiculus(second segment) and club (third segment), black compound eyes, black color, small white spots scattering on elytra,body length 5.06 mm, wing with longitudinal grooves, narrow at anterior end and broader at posterior end of a black pronotum with white spots all over it, black rostrum slightly curved, pronotral length 1.52 mm, 3 pairs of leg appendages covered with elongated white setae directed downward, foreleg femur with a triangular tooth (Figure 1), forewing length 2.13 mm, membranous blackish hindwing, dorsum of the 2nd and 3rd thorax and abdomen dark brown, ventral abdomen black (Table 2).

Description of Female

Similar to male characteristics, slightly larger than males (Table 2), body length 5.36 mm long, rostrum 1.79 mm long, forewing 2.40 mm wide and 4.31 mm long, hindwing 2.60 mm wide and 7.51 mm long.

Table 1 Dimension in millimeter of different life stage of *Cionus* sp.

Stage of insect	width	length
Egg	0.48±0.1	0.86±0.04
1st instar	1.01±0.06	1.89±0.20
2nd instar	1.79±0.04	3.46±0.57
3rd instar	3.21±0.49	6.62±0.84
pupa	3.37±0.16	4.80±0.20

Table 2 Dimension in millimeter of adults

Size	male	female
Body length	5.06±0.08	5.36±0.06
Rostral length	1.52±0.04	1.79±0.03
Forewing width	2.13±0.04	2.40±0.01
Forewing length	4.02±0.06	4.31±0.02
Hindwing width	2.50±0.01	2.60±0.01
Hindwing length	7.50±0.02	7.51±0.02

Figure 1 A triangular tooth on a foreleg femur

Life History of Dolichanndrone Weevil

Adult emerged from cocoon in the morning. Mating occurred after adult aged for 5-7 days. Copulation can last for 6-10 hours during day and night and several times (Figure 2). The female laid eggs in group of 3-7 egg/groove on a young petiole (Figure 3) and it could produce on an average of 109 eggs in a lifetime. The female of *C. tuberculosus* spent 90 mins for groove building, oviposition and cavity sealing (Rather, 1989: Read,1977). It took 3-4 days for a first instar larva to hatch (Table 3). There were 3 immature stages of larva which lasted for 6.26 days. These larvae fed on young leaves. Larvae are slug-like and Cunningham (1979) stated that the muscous, hyaline covering of the insect larvae could protect them from desiccation. When the larvae were fully grown, it would pupate at the leaf surface of host plants (*D. serrulata*). The last instar larvae would make up the cocoon during the night, one cocoon/leaf. The pupal period was 5.19 days. Adult emergence from cocoon mostly in the morning using rostrum to make a wider hole for exit (Figure 4). When being disturbed, adults turned death-feigning. Adult's lifespan under laboratory condition was 82.35 days for the female and 81.20 days for the male. The results agreed with Peng et al.(2009) on *C. latefasciatus* female's life expectancy longer than the male. Males develop faster because of they are smaller, whereas females had longer development due to larger bodies and increase fecundity by more feeding (Dank, 2000). In nature, both larvae and adults are commonly found in rainy season. The female can lay eggs up to 60-152 egg for its lifespan.

Figure 2. Mating copulation of *Cionus* sp.

Figure 3. Egg deposition of *Cionus* sp. in a young petiole

Table 3 Duration time of *Cionus* sp.

Developmental stage	n	Duration time(Day)	
		mean±SD	range
Egg	30	3.26±0.43	3.00-4.00
Larva	30	6.26±0.38	5.75-6.83
Pupa	30	5.19±0.19	4.50-5.55
Adult			
male	20	81.20±41.85	35-180
female	20	82.35±28.63	40-154

Infestation of Dolichandrone weevil

Larvae feed on the ventral part of young leaves and surface of young twigs near the young buds(Figure 5). Adults chew irregular shaped holes all over young and old leaves of host plants(Figure 6). The damaged leaves would dry up and turn brown. During egg laying, the female use a snout to make a groove on young petiole for egg deposition and result in leaf petiole folded down and wilted. Damage is caused by both larvae and adults of *Cionus* sp from May to November when large number of all active stages appeared on their host plants.

Parasitization of *Entedon* sp. on Dolichandrone Weevil

Entedon sp. is an important parasitoid of Dolichandrone weevil(figure 7)**.** It is a small parasitoid which the males is smaller than the female(1.80 and 2.40 mm, respectively). Sexual fitness might be correlated with body size (Ode and Heinz, 2002). Females of *Entedon* sp. oviposit their eggs into various instar larvae and cocoons of *Cionus* sp. Parasitization rate of this eulophid parasitoid species on the instar larvae and pupa of Dolichandrone weevil which were collected from the field was assessed. The parasitization occurrence during May, 2017 to July, 2018 was 6.25-24.19% in larvae and 0.00-62.50 % in papa (Table 4). The laboratory data indicated that sex ratio of *Entedon* sp (male to female) is between 1: 0.11 and 1: 3.64 (n=21).

Figure 4. Fully matured *Cionus* sp.emerged from the cocoon

Figure 5. Larvae feed on surface of a young twig

Figure 6. Adults chew on *D. Serrulata*'s leaf

Figure 7. *Entedon* sp.

Table 4 *Cionus* sp. attacked by *Entedon* sp. in 2017-2018

Collection date	*Cionus* larva		*Cionus* pupa	
	n	parasitization(%)	n	parasitization(%)
May 2017	84	19.05	5	40.00
June 2017	61	19.39	3	33.33
July 2017	96	6.25	4	50.00
August 2017	62	24.19	8	62.50
September 2107	53	16.98	6	50.00
November 2017	70	8.57	12	16.67
May 2018	102	21.57	9	33.33
June 2018	47	8.51	2	0.00
July 2108	12	16.67	2	50.00

Conclusion

The Dolichandrone weevil is a small sized insect in the family Curculionidae, order Coleoptera. Both adult and larvae are heavy leaf feeder of *D. serrulata*. In addition, it caused multiple lesions on the leaf stalk a petiole folded and young leaf at the tip of the petiole wilt and dieback. Dolichandrone weevils are easily regconized by their unique white spots scaterring all over the pronotal dorsum and elytra. The adult did faking death to protect itself from its predators. All stages of larval instar are slug-like and fully grown larvae build cocoons for pupation. Their successive immature stages has a relatively short lifespan as being compared with the adults The females are slightly bigger and live longer than the male. Both adults and larvae of *Cionus* sp live in the same habitat. *Entedon* sp is commonly found attacked on both larva and pupa of *Cionus* sp. from May to November. Moreover, the method of mass rearing *Entedon* sp. in the laboratory could be further studied..

Recommendations

D. serrulata is an ornamental plant grown in residential areas and public parks, thus it is not safe to use insecticides to control the Dolichandrone weevil. The precaution should be taken when the larvae were frist found during field survey to prevent long lived adult emergence. Therefore weevil trap and a biological control agent with *Entedon* sp. would be fit for the control management.

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New Infection Model for Foliar Fungal Plant Pathogens

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Abstract: Epidemiological models for plant diseases are very important for prediction, control and estimation of the infection incidence. Such models are fundamentally characterized by the influence of some meteorological variables. In this paper, we design a new model to predict incidence of infection by pathogens in function of the mixed effects of temperature and wetness. These deeply influential parameters are estimated and adjusted with regards to the disease caused by various infectious pathogens. In addition, we show that it is primordial to introduce bound constraints on the model's location parameters. This allows to perform a more accurate minimization of the sum of residuals. The proposed optimization procedure is based on the trust-region method. Our methodological approach is simple and easy to implement for the prediction and / or control of diverse plant infections. In order to show its efficiency, our model is validated and compared for different plant diseases adapted from several studies published in the literature. As a matter of comparison, the results of goodness of fit demonstrate that our new model outperforms the other reported models.

Keywords: Plant disease, Epidemic Models, Temperature and wetness duration, Fitting, Nonlinear optimization

Introduction

The design of disease models for plant infections is the only rigorous way for disease forecasting and control (Delignette-Muller 2009; Krause and Massie 1975). In this spirit, the infection propagation must be adequately described by mathematical models that are conceived in order to express the interaction between some environmental variables that can be measured and studied in laboratory, greenhouse, field experiments or simulated (Krause and Massie 1975; Madden and Ellis 1988; SHRUM 1978; Waggoner 1974). Note that, in the areas of crop physiology and agricultural meteorology the temperature, the surface wetness, the humidity and the rainfall, are the most commonly considered environmental variables (Wang and Engel 1998; YAN and HUNT 1999; Yin et al. 1995).

In this paper, we consider two important issues: The design of a suitable infection model for plant pathogens, and the foundation of an original methodology for fitting. Specifically, we provide a model that can be efficiently applied to plant infections with regard to the mixed effects of the temperature and the wetness duration. Moreover, our model has good merits compared with other published models (Duthie 1997; Furuya et al. 2009; Grove, Madden, and Schmitthenner 1985; Sharma et al. 2014). One of these merits is that its whole parameters have an intrinsic biological meaning, which describes the scale, the shape, and the location of the disease response.

A second merit concerning our approach is that in contrast to many other epidemic models reported in the literature for which the location parameters are predetermined or empirically fixed or even absent in the model (Duthie 1997; Erincik et al. 2003; Sharma et al. 2014); these fundamental biological parameters are considered as unknown variables that should be determined from the fitting procedure. As a result, based on the introduced methodology, the proposed model is applied to various plants and infectious pathogens with comparison to some important well-known models in the literature (Furuya et al. 2009; Grove et al. 1985; Sharma et al. 2014). Moreover, it is shown that our original least-square procedure with bound constraints

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introduced in this paper, leads to efficient and better fitting for three kinds of plants with different fungal infections based upon experimental data sets. In all the case studies, our model is shown to be very competitive and outperforms the well-known reported models.

The reminder of the paper is organized as follows. In Section 2, we introduce the epidemic model for plants infection under mixed effects of temperature and wetness. Section 3 provides a fitting method for the parameters estimation. In section 4, the proposed models are validated using simulation results with comparison to some reported works in the literature. Finally, concluding remarks are presented in Section 5.

Plant Diseases Model

We consider a mixed effects modeling for plant disease with regard to the relative infection response subject to the effect of the most influential environmental variables represented by the temperature and the wetness duration.
The model is descried by:

$$y(t,w) = f(t,w) + \varepsilon \tag{1}$$

Where the function f is expressed as

$$f(t,w) = \left(1 - \frac{1}{((h_1(t-t_I)^{f_1})+1)}\right)\left(1 - \frac{1}{((h_2(t_C-t)^{f_2})+1)}\right)\left(1 - \frac{1}{(h_3(w-w_I)^{f_3})+1}\right) \tag{2}$$

The involved quantities under study are

- y is the measure of the relative infection on a scale from 0 to 1 (or score response tacking values in the interval [0 ; 1]).
- t is the temperature (in Celsius).
- w is wetness duration (in hours).
- ϵ is the model residual or perturbation error represented by an unknown random normal variable

The parameters of the model are defined by

- h_1, f_1, h_2, f_2, and h_3, f_3 are the shape paramerters.
- t_I, t_C, and w_I are the location paramerters such that
  - t_I : minimum temperature required for infection (in Celsius)

$t_I = \min(t \mid y \neq 0)$

  - t_C : *maximum temperature required for cure (in Celsius)*

$t_C = \max(t \mid y \neq 0)$

  - w_I: *minimum wetness duration for infection (in hours)*

$w_I = \min(w \mid y \neq 0)$

It is worth mentioning that by construction of the model, the location parameters intrinsically satisfy the fact that for all (t,w) we have $y(t_I,w) = y(t_C,w) = y(t,w_I) = 0$.

Proposition 1.

The function f take its values in the interval $[0,1]$ if and only if $h_1>0, h_2 > 0, f_1 > 0, f_2 > 0, f_3 > 0, h_3 > 0$.

Proof: The condition $h_1>0, h_2 > 0, h_3 > 0, f_1 > 0, f_2 > 0$, and $f_3 > 0$ results from the fact that the functions f do not go to infinity at the boundary of the domain $[t_I\ t_C] \times w_C$

Parameters Estimation

The proposed parameters estimation for our model is based on likelihood principle which under some statistically legitimate assumptions (independence of measured values, normal distribution of the residual), this leads to fit a given model to the observed data based upon the least square procedure.

In the sequel, we consider the sample data of measured temperature $T := \{t_1, \dots\dots\dots, t_n\}$ and the set of measured wetness duration $W := \{w_1, \dots\dots\dots, w_n\}$ with their corresponding observed relative infection $Y := \{y_1, \dots\dots\dots, y_n\}$. Then, in order to fit the model to the sample data Y by estimating the involved parameters, one has to find the best parameters $h_1, f_1, h_2, f_2, h_3, f_3, t_I, t_C, w_I$ that are effectively the optimal solution to the following nonlinear least square optimization (Sum of Squares Error (SSE) minimization)

$$SSE := min_{h_1,f_1,h_2,f_2,h_3,f_3,t_I,t_C,w_I} \|y - \hat{y}\|^2 \tag{3}$$

The estimate model $\hat{y} = f(t, w)$ is entirely described by the function f;

The above optimization problem can be solved more efficiently if one can include some information from the sets T and W. In fact, the useful information is to take into account the following underlying constraints

$$\begin{cases} t_C^- \leq t_C \\ t_I \leq t_I^+ \\ w_I \leq w_I^+ \end{cases} \tag{4}$$

Hence, we add natural bounds from the data sets by integrating the lower bound on t_C and the upper bounds respectively on t_I and w_I.

In the following, it is show how to obtain the previous introduced bounds.

Proposition 2. In order to fit the data sets $(Y,\ T, W)$, the following bounds must hold

$$\begin{cases} t_C^- := max_{y_i \neq 0}(t_i) \\ t_I^+ := min_{y_i \neq 0}(t_i) \\ w_I^+ := min_{y_i \neq 0}(w_i) \end{cases} \tag{5}$$

Proof. The argument line is straightforward from the construction of the model.

Now, considering **Proposition 1**, the constrained nonlinear least square optimization problem under consideration for fitting the proposed model to the sample data $(Y,\ T, W)$ is adequately formulated as:

$$min_{h_1,f_1,h_2,f_2,h_3,f_3,t_I,t_C,w_I} \|y - \hat{y}\|^2 \tag{6}$$

Subject to

$$\begin{cases} h_1 > 0, f_1 > 0, h_2 > 0, f_2 > 0, a > 0, b > 0 \\ t_C^- \leq t_C \leq t_c^+ \\ t_I^- \leq t_I \leq t_I^+ \\ w_I^- \leq w_I \leq w_I^+ \end{cases} \tag{7}$$

Were, one can set t_C^+, t_I^- and w_I^- to some empirical or trivial meteorological extremum values. For instance, set $w_I^- = 0h$ and more or less $t_C^+ = 40\ C°$, $t_I^- = 0C°$.

This constrained optimization problem can be solved using nonlinear programming methods such as the classical Gauss-Newton with its many variants, Trust-Region, Interior Points method. These methods work well in practice, if the initial starting points are well-guessed or can be close to the optimal solution.

Models Validation and Numerical Results

Here, we perform a comparison study with the most well-known nonlinear models related to fungal infection that are based upon the combined effects of wetness duration and temperature.
These models can be categorized as follows

- Beta model :

$$f(t,w) = a(t-t_I)^b(t_C-t)^c w^d \quad (8)$$

- Duthie's model :

$$f(t,w) = \frac{e(h+1)h^{((h-1)^{-1}-1)}exp(g(h+1)^{-1}(t-f))}{1+exp(g(t-f))} \quad (9)$$

- Polynomial model :

$$log\left(\frac{y}{1-y}\right) = a_0 + w(a_1 + a_2 t + a_3 t^2 + a_4 t^3) \quad (10)$$

In order to fit our model, we have applied Trust Region method to solve the optimization problem. Matlab implements this method via the function fit. The chosen initial conditions t_I^0, t_C^0 and w_I^0 for the temperature and the wetness, are close to the proposed bounds on t_I, t_C and w_I. For the others initial parameters we have selected values between 0 and 1.

In the simulation results, we have considered SSE index accuracy, which indicates close estimates to the real observed disease values y.

Note that the goodness of the fit greatly depends on the statistical measures of performance. Hence, we have also validated our model via the well-known statistical goodness of fit indicators R^2 and its adjusted valueR_a^2, which show the tendency to the linear relationship between the predicted and observed values as they become closer to the 1 (perfect fit).

The simulation results in Tables 1.and 2. Show that the two proposed models are more effective than the other reported models (8)(9)(10) . It can be seen that our performance factors are better since they provide a better fit and significantly outperform these alternative models.

Table 1. Different infection model based on published studies relating fungal infection to temperature and wetness duration

				Goodness of fit			
Pathogen	Host	Model	Ref	R^2	R_a^2	SSE	$RSSE$
Powdery mildew	Picrorhiza kurrooa	Beta	(Analytis 1977; Erincik et al. 2003; Mio and Amorin 2002; Sharma et al. 2014)	0.81	*	*	*
Puccinia allii	Spring onion	Dutie	(Duthie 1997; Furuya et al. 2009; Wu et al. 1999)	0.9501	*	*	*
Spinach White Rust	spinach	Polynomia l	(Madden and Ellis 1988; Sullivan, Damicone, and Payton 2002)	0.8917	0.8909	*	*

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Table 2. Parameters estimation for the model (2) with their corresponding goodness of fit

			Parametre									Goodness of fit			
Pathogen	Host	Ref	h_1	h_2	h_3	f_1	f_2	f_3	t_I	t_C	w_I	R^2	R_a^2	SSE	$RSSE$
Powdery mildew	Picrorhiza kurrooa	(Sharma et al. 2014)	7.25e-08	3.31	0.009	10.78	866.3	1.84	29	38	0.1	0.9687	0.9521	0.0893	0.077
Puccinia allii	Spring onion	(Furuya et al. 2009)	0.4417	0.03653	1.602	2.1	6.002	3.4	1.872	27.38	0.6469	0.9549	0.9308	0.1728	0.1073
Spinach White Rust	spinach	(Sullivan et al. 2002)	1.74e-4	0.00134	1.03e-3	5.35	2.97	2.953	4.285	30.65	1.3e-4	0.9003	0.8913	0.7510	0.0919

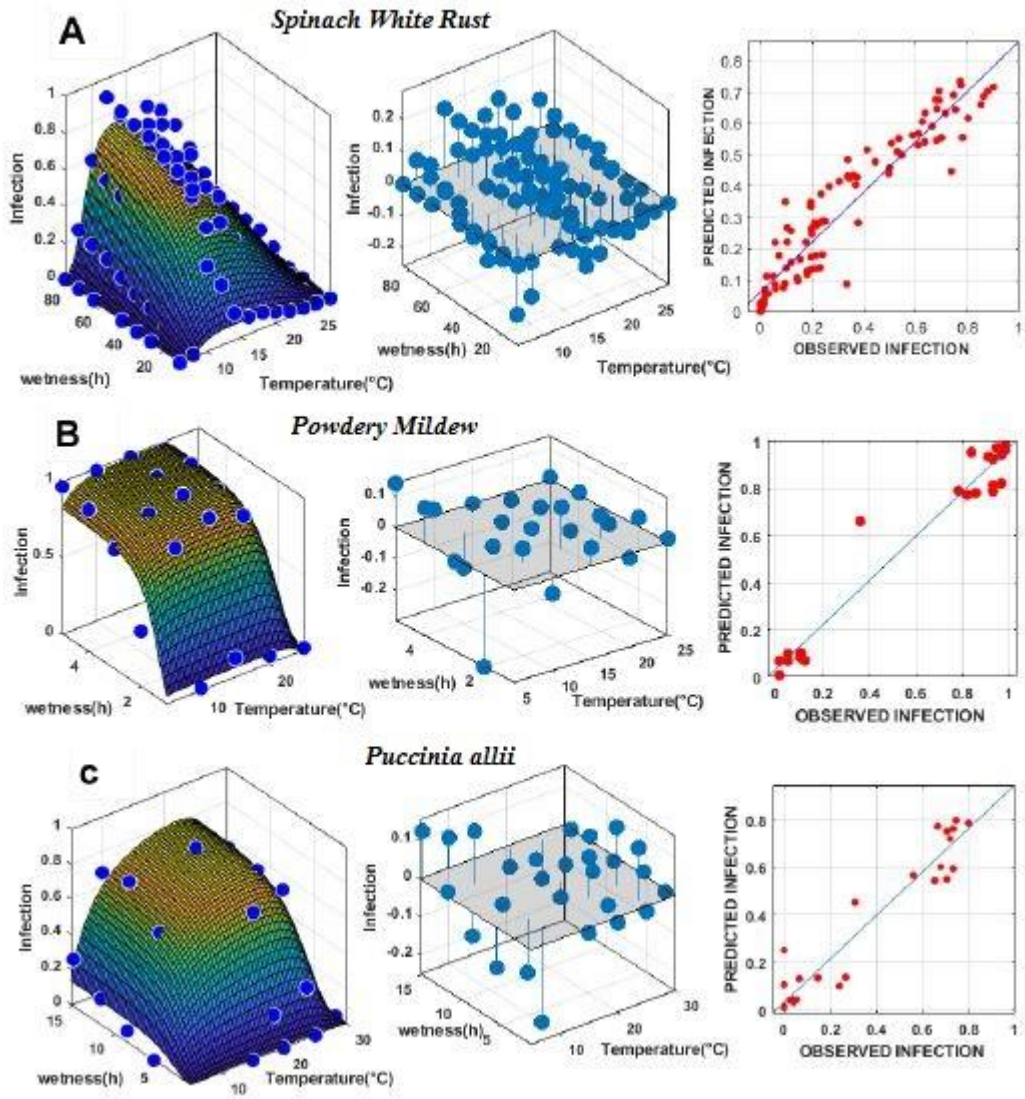

Figure 1. Observed and predicted values of the relative disease using model *(2)* and Residuals versus the fitted values

Conclusion

The model and the approach provided in this paper are original. It has been shown that accurate modeling for plant disease can be achieved based upon the provided techniques, which can be implemented for simulation analysis, prediction and/or control. Moreover, the comparison study has shown the efficiency of the proposed epidemic model.

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Ge-NOSE: Electronic Nose for Sniffing Food-Borne Bacteria

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Abstract: Gastronomy practice has become major attraction in tourism also promote food importation globally. So, controlling bacterial contamination to comply biosecurity regulations is one of imperative task for quarantine services. However detection method of bacteria causing food poisoning is laborious. Electronic nose technology has ability to recognise volatile compounds (VOCs) emitted by biological materials. Recently, the *Elecetronic Nose* is one of the best choice since it does not need reagen, cheap and fast. To proof-of concept, an investigation was carried out employing Ge-Nose (Universitas Gadjah Mada, Indonesia) to captured volatile emission of four food-borne bacteria: *E.coli* (ATCC 25922), *S.thypimurium* (ATCC 14028), *L.monocytogenes* 4b (ATCC 13932) and *B.cereus* (ATCC 10876). All of sample were then incubated at 37°C for 2, 8, 16, 24, 32, 40, and 48 hours then analysed using different methods such as *Linear Discriminan Analysis* (*LDA*), *Quadratic Discriminant Analysis* (*QDA*), and *SupportVectorMachine*(*SVM*). The result showed, using LDA methods, accuracy value of *E.coli was* 97.80±2.20%; *S.thypimurium:* 94.60±5.40%*: ; L.monocytogenes* 98.00±2.00%*:* and *B.cereus* 95.00±5.00%. Using *QDA methods, the accuarcy value of E.coli was* 94.80±5.20%; *S.thypimurium:* 95.60±4.40%*: ; L.monocytogenes* 92.00±8.00%*:* and *B.cereus* 95.00±5.00%; whereas SVM methods, it has been showed: *E.coli was* 97.00±3.00%; *S.thypimurium:* 92.40±7.60%*: ; L.monocytogenes* 89.00±11.00%*:* and *B.cereus* 89.00±11.00%. Highest accuracy classification average (98%) was achieved. Therefore, Ge-NOSE's discriminate power is able to deliver faster, accurate yet simple and inexpensive diagnostic result.

Keywords: Electronic Nose, *E.coli*, *S.thypimurium*, *L.monocytogenes*, *B.cereus*

Introduction

Food-borne disease has become serious issue for public health and food safety. However identification and detection of pathogen bacteria from clinical samples, environment or food is time-consuming (Gates, 2011). Other detection method such as Gas chromatography Nuclear Magnetic Resonance (NMR), Spectroscopy and Fourier Transform Infrared (FTIR) is high-cost besides required trained operator (Adley, 2006;Tian *et al*., 2013). Test that is rapid yet simple becoming increasingly important (Yu dan Zhao, 2012; Tait *et al*., 2014) and electronic nose (EN) provides promising features to overcome diagnosing limitations.

Electronic nose is odours-sensor-based device featuring beneficial attributes to help delivering faster, accurate, simple and inexpensive diagnostic result. The EN has been used in veterinary field to detect oestrus cycle in cow and recognise species of animal in mincemeat. However this discriminate power of EN has not been explore to identify food-borne bacteria. Therefore, this research is carried out to detect four significant bacteria causing food poisoning: *Escherichia coli, Salmonella thypimurium, Listeria monocytogenes*, and *Bacillus cereus*. The total number of those bacteria must comply strict regulations of Indonesian National Standard (SNI).

Bacteria are producing volatile organics compounds (VOCs) and gas specifically to each species, which able to be sniffed by EN sensors. Captured VOCs then analysed by pattern recognition chemomatric (PARC) (Capone *et al.*, 2001, Evans *et al.*, 2000, Haugen and Kvaal, 1998) and classified.

Material and Methods

Four ATCCs bacteria: *E.coli* (ATCC 25922), *S.typhimurium* (ATCC 14028), *L.monocytogenes* (ATCC 13932) and *B.cereus* (ATCC 10876) were cultured in medium at 37°C for 2, 8, 16, 24, 32, 40, 48 hours. Electronic nose Ge-NOSE 4th Generation (Fismatel, Physics Department, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Indonesia) was employed to captured emitted VOCs. The Ge-NOSE was equiped with TGS 2600 (Air Quality Sensor), TGS 2603 (Odorous Gas Sensor), TGS 2612 (Methane and Liquefied Petroleum Gas Sensor), TGS 2620 (Solvent Vapors Sensor), TGS 813 (Combustible Gas Sensor), TGS 822 (Organic Solvent Vapors Sensor), TGS 826 (Ammonia Sensor), and TGS 832 (Chlorofluorocarbon Sensor).

To start, Ge-NOSE was undergone initial flushing for 10 minutes. Samples were warmed up between 37-47^{0}C for 70 seconds to produce maximum bacteria metabolites allowing maximum captured by the array sensors at 0.1 s/data speed. Each sample was sniffed for 1 minute and each sampling lasted 10 seconds. Flushing for 1 minute was applied between sample measurements.

Captured VOCs pattern was analysed by pattern recognition (PARC) then classified by Linear Discriminant Analysis (LDA), Quadratic Discriminant Analysis (QDA) and Support Vector Machine (SVM)

Ethics Statement

The research is exempt from full ethical clearance process based on no use of animals.

Result

In order to equally comparing the data, VOCs captured by each sensors were normalised prior analysis (Figure.1). Classification analysis by LDA (Figure.2A) showed clear clustering of contaminated medium by single bacteria (blue) compared to non-contaminated medium (red).

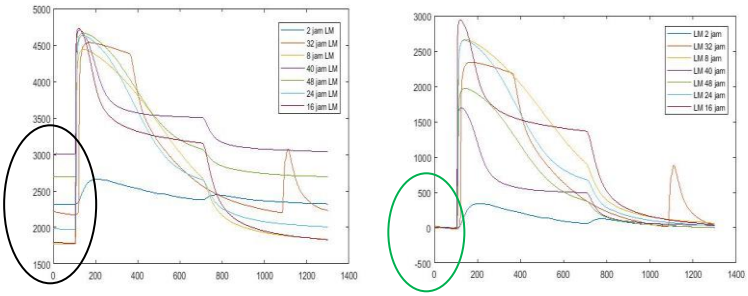

Figure 1. A. Sensor TGS 822 response to *L.monocytogenes* incubated at 2, 8, 16, 24, 32, 40 and 48 hours prior baseline normalisation (black circle), B. After baseline normalisation (green circle) should be started from 0 (source: Astantri. 2019)

The existance of *E.coli, S.typhy, L.monocytogenes*, and *B.cereus* in the medium was detected by all equipped sensors in Ge-NOSE. Chemomatric analysis of LDA, QDA and SVM was able to discriminate non-contaminated and contaminated medium either with single or multiple bacteria under different level of accuracy (Table 1). LDA classification showed highest average accuracy of 98% in comparisson to QDA and SVM, in distinguish non-contaminated and single-bacteri contaminated media. Monocytogenes was showing highest accuracy (98%) whilst Thypimurium the lowest (94.6%).

Table 1. Accuracy level between LDA, QDA and SVM method in classifying non-contaminated and contaminated medium (Prakoso., 2019; Astantri., 2019)

Group	*LDA*		*QDA*		*SVM*	
	Accuracy (%)	Std	Accuracy (%)	Std	Accuracy (%)	Std
N vs E	97.80	2.20	94.80	5.20	97.00	3.00
N vs S	94.60	5.40	95.60	4.40	92.40	7.60
N vs L	98.00	2.00	92.00	8.00	89.00	11.00
N vs B	95.00	5.00	95.00	5.00	89.00	11.00

Note: N: Negative control, E: *Escherichia coli*, S: *Salmonella thypimurium*, L: *Listeria monocytogenes*, B: *Bacillus cereus*. Std: Standart deviation

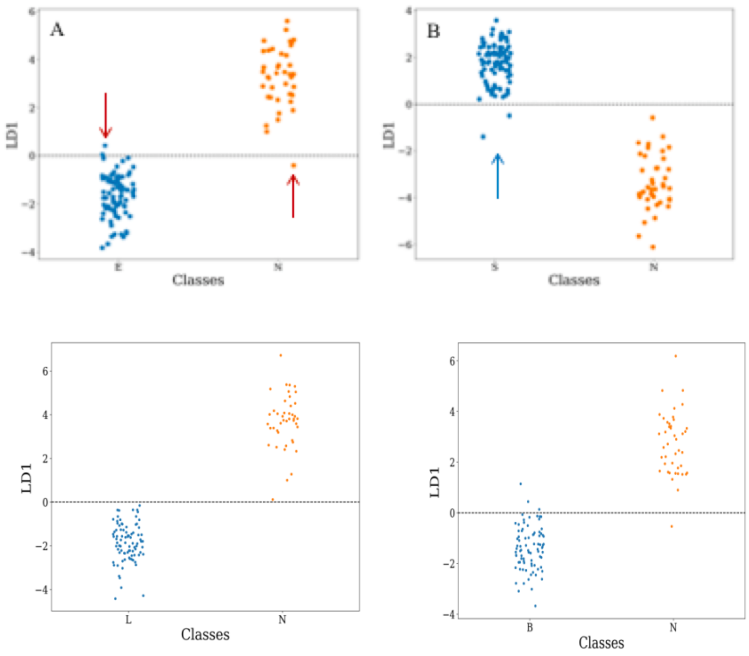

Figure 2. A. Clustered of *E coli* bacteria (blue) and clustered of non-contaminated medkum (red) with 97.8% of accuracy level. B. Clustered of *S. thypimurium* bacteria (blue) and clustered of non-contaminated medkum (red) with 94.6% of accuracy level (source: modified Prakoso., 2019)

Discussion

L.monocytogenes produced alcohol, aldehyde, ketone and alkane (Yu *et al.*, 2015) with predominance VOCs 3-methyl-butanal (Arnold & Senter, 2012), 2-nitrophenol and 3-fluoroaniline (Tait, 2014) whereas *B.cereus* produced 3-methyl-1-butanol, aldehyde, acetyl acid, and ethanol (Yu *et al*.,, 2015). These VOCs produce by the bacteria will enable application of EN for detection.

The level of accuracy between chemomatrics analysis is showing the influence of bacteria type and growth time, time of storage and chemomatrics method used. Rosyad and Lelono (2016) reported highest response of electronic nose sensors to VOCs at 16 hours of incubation. Bacteria VOCs emission may be reduce during supressed growing phase due to poor quality of media, altered medium pH, metabolite waste accumulation and overgrowth (Dwidjoseputro, 1998).

Therefore the result of this study is clearly indicating the accuracy of Ge-NOSE in detecting *Escherichia coli, Salmonella thypimurium*, *Listeria monocytogenes* and *Bacillus cereus* in medium.

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