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PHYTOCHEMICAL EVALUATION AND PHARMACOLOGICAL ACTIVITIES OF *ANTIDESMA MONTANUM* BLUME LEAF EXTRACT

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Abstract: The demand for medicinal plants and their derived substances is increasing day by day due to their relevance in the context of drug discovery and development. The goal of this investigation is to assess the pharmacological and phytochemical potentials of the grossly underexplored *Antidesma montanum* Blume (Family: Phyllanthaceae). The methanolic extract of the leave of this plant was fractionated and then followed by initial screening of phytochemical. The investigation of the pharmacological potential, which includes antioxidant, antidiarrheal, anti-inflammatory, analgesic, anti-pyretic, and anxiolytic evaluations, was accomplished using an *in vitro* free radical scavenging assay with 2,2-diphenyl-1-picrylhydrazyl (DPPH), castor oil-induced diarrheal test, egg albumin test, acetic acid-induced writhing model, brewer's yeast induced fever test, swing test, open field, and light-dark test, respectively. The investigation o phytochemicals proposes that the methanol extract of *A. montanum* possesses flavonoids, tannins, terpenoids, saponins, amino acids, fixed oils, and sterols. Pharmacological evaluation suggests that *A. montanum* possesses significant antioxidant, anti-diarrheal, anti-inflammatory, and analgesic effects. The methanol and chloroform fractions exhibited better DPPH radical scavenging activities with an IC_{50}: 103 ± 0.05 and 108.7 ± 0.05 µg/ml, respectively. The methanol and chloroform fractions also showed anti-inflammatory capacities in the egg albumin (IC_{50} values: 89.10 ± 0.07 and 92.85 ± 0.07 µg/ml, respectively) model. The plant also showed anti-pyretic and anxiolytic activities in a dose-dependent manner. One of the possible sources of phytotherapeutic lead compounds is *A. montanum*. To extract and analyze the key bioactive components of this essential therapeutic plant, more research is required.

Keywords: *Antidesma montanum*; phytochemicals; pharmacological activities.

1. Introduction

Conventional medicines rely on phytochemical-rich plant extracts to cure different maladies because medicines obtained from natural sources are considered to be less toxic and free from undesirable effects as compared to synthetic ones. The genus *Antidesma*, for example, contains over 200 species, with *Antidesma montanum*

Blume is being the most common (Ismail et al., 2019).

A. montanum commonly referred to as mountain current tree or pani-helochis a shrub or trees belong to the family Phyllanthaceae. *A. montanum* species may be found in a broad range of environments from the coast to the highland, from profound forest shade to surrounding human habitations and open grassland, and at altitudes up to 2,000 m. It is distributed in East Asia – Bangladesh, India, China, Myanmar, Vietnam, Cambodia, Indonesia, Malaysia, and Thailand. The plant may reach a height of 20 m. For up to 7 m, the bole can be free of branches. Alternate, stipulate leaves with lateral stipules measuring 3-12 x 0.5-3 mm. Flowers are yellow and unisexual. The fruit is a drupe that is 3-6 x 2.5-4 mm and is ellipsoid to ovoid in shape (https://indiabiodiversity.org/species/show/7553). Internally, *A. montanum* roots in water have been used to cure malaria, chickenpox, and measles in Peninsular Malaysia, while the leaves have been used topically to treat thrush and headache in children. Stems and roots are used as a diuretic in Thailand. The roots are used in the treatment of stomach ache (Ismail et al., 2019). In South-East Asia, the leaves of other *Antidesma* species are also utilized medicinally. After giving delivery, moms drink a tea made from the leaves as a tonic. Topically, the leaves are used to treat lumbar aches and ulcers (http://tropical.theferns.info/viewtropical.php?id =Antidesma + montanum). The plant contains essential oils that is rich in n-hexadecanoic acid (Zhou et al., 2012a; Zhou et al., 2012b); alkaloids, steroids, anthquinones, saponins, phytophenols, flavonoids and tannins (Arbainand, 1993; Aguninaldoet al., 2004).

The methanol extract of *A. montanum* leaves has potential antioxidant activity capacity (Maehly, 1954). The current study examines the phytochemical and pharmacological evaluation of a leaf extract of *A. montanum* which has not been hitherto properly investigated.

2. Materials and methods

Collection, identification, and extraction

Zhengzhou Fresh leaves of *A. montanum* were taken from a forest at Kamalgonj in Bangladesh, for this study, and were recognized by a taxonomist at Jahangirnagar University's Department of Botany. The plants were placed on a herbarium sheet, and the Bangladesh National Herbarium in Mirpur, Dhaka, identified the sample (Accession number-46870, DACB). The leaves were gathered, sorted from unwanted plants or plant components, and washed in water. They were dried in the shade for one week at room temperature (25-35 ºC). With the aid of a competent grinder, the leaves were crushed into a coarse powder. Until the analysis, the powder was maintained in an airtight container in a dark, cool, and dry environment. 350 gm of energized material were soaked in 1800 ml of methanol (99-100%). For a period of 15 days, the container and its contents were sealed and preserved with some shaking and stirring in between. After that, a piece of clean white linen was used to roughly filter the entire mixture. Finally, the extract was filtered using filter paper. The methanol extract was obtained by centrifuging the filtrate with a rotary evaporator. The resulting extract was kept in a beaker covered with a perforated sheet of aluminum foil for several days to ensure complete evaporation of the residual methanol. It resulted in a sticky concentration with a greenish-black tint. Crude methanol extract was the name given to the

concentration. By following the same process, a chloroform extract of *A. montanum was* also prepared.

Reagents and chemicals

ACI Pharmaceuticals Ltd. provided the diclofenac sodium and paracetamol, while ACME Laboratories Ltd. provided the diazepam and loperamide. Gonoshasthaya Pharmaceuticals Ltd. delivered the yeast. Merck Chemicals Ltd., Germany, provided the acetic acid and Tween-80, while sterile normal saline (0.9% NaCl) was provided by Beximco Infusions Ltd. The solvents and chemicals used were all of analytical quality. On the day of the experiments, all of the solutions were made.

Experimental animals

The animals utilized were *Swiss* albino mice measuring 20–25 gm. During the acclimation phase, the animals were maintained in regular laboratory settings (12 h light/dark cycle; room temperature 25 ± 2 °C; relative humidity 55–60%) and fed a standard diet (ICDDR, B prepared) and distilled water ad libitum. Prior to executing the studies, the animals were acclimatized to the laboratory environment for 7 days. Before the studies, the animals were starved for the night. All experimental animals were cared for in accordance with the Swiss Academy of Medical Sciences and the Swiss Academy of Sciences' Ethical Principles and Guidelines for Scientific Experiments with Animals (1995). The Institutional Ethics Committee (SUB/IAEC/12.01) authorized all experimental protocols.

Preliminary phytochemical screening

The preliminary phytochemical investigations consist of testing distinct phytochemical classes contained in the extract. For this test, two types of extract solutions were prepared: a 5% w/v alcoholic extract solution (1.5 gm diluted in 30 ml ethanol) and a 5% w/v aqueous extract solution (In 2 ml of pure water, 0.1 gm was dissolved) (Harbopne et al., 1973; Evans et al., 1989; Edeoga et al., 2005).

Acute toxicity evaluation: Allium cepa assay

The crude methanol extract of the plant leaves was examined at 4.0, 3.0, 2.0, 1.0, and 0.5 mg/ml using distilled water as a control group. To stimulate root growth, the budding parenchyma and outer layers of the onions were carefully removed via the creation of a tiny circular incision. Generally, the tap water is applied for 20 min to clean the bulbs, while distilled water is used for soaking the root section in a cleaned container (15-20 ml capacity) at the condition of 25 ± 1 ^{o}C in the dark for the first 24 h. For a 24-hour exposure period, only the Alliums with adequate root development were replaced to the sample or controls containers containing five for each concentration. The roots were measured in mm after the exposure time. Root growth inhibition was determined to estimate the toxicity of the crude extract. The test sample's IC_{50} was also determined by applying following equation:

$$\log (LD_{50}) = 0.435 \times \log (IC_{50}) + 0.625$$

Antioxidant capacity

Total phenolic content assay

0.5 g dried material of plant was sonicated for 20 minutes with 50 ml methanol. The extract was centrifuged for 15 minutes after being divided into two liters. The standard, 30 mg of gallic acid, was combined with 250 ml of 99-100 percent methanol to make a 200 g/ml solution. Furthermore, using the right dilution process, four concentrations of solutions (150, 100, 50,

and 25 g/ml) were created, and volumetric flasks were taken and labeled. 1 ml of each concentration's standard solution was added to volumetric flasks, along with distilled water (9 ml) and 1 ml of Folin-Ciocalteu (FC) reagent, which was shaken and maintained for 5 minutes. The volume was adjusted with distilled water to reach a final amount of 25 ml after adding a 10 ml solution of 7% Na_2CO_3. They were maintained at room temperature for 90 minutes.

For each concentration, the UV absorbance was measured at 750 nm after 90 minutes against a blank. For accuracy, the absorbance was measured three times and the mean was chosen.

Total tannin content assay

0.5 g dried material of plant was sonicated for 20 minutes with 50 ml of 80% aqueous methanol. Two ml of the extract were centrifuged for 15 min. Gallic acid (30 mg) as a standard was mixed with 250 ml of methanol (99-100%) to prepare a 200 µg/ml solution. Another four concentrations of solutions were prepared by the proper dilution method. The concentrations were 150, 100, 50, and 25 µg/ml, respectively. 200, 150, 100, 50, and 25 µg/ml volumetric flasks were used, and standard solution (0.1 ml) was added to the flasks, followed by distilled water (7.5 ml), and 0.5 ml of FC reagent, which was held for 5 minutes. Then 1 ml of a 35% Na_2CO_3 solution was added, and 10 ml with distilled water was poured to adjusted the volume and maintained at room temperature for 30 minutes. For each concentration, the UV absorbance was measured at 725 nm against a blank. For accuracy, the absorbance was measured three times and the mean was chosen.

Total flavonoids content assay

A total of 0.5 gram of dried plant material was combined with methanol (50 ml) and sonicated for 20 minutes. Two (2) ml of the extract were centrifuged for 15 min. The standard, quercetin (30 mg), was mixed with 250 ml of methanol (99-100%) to prepare a 200 µg/ml solution. Another four concentrations of solutions (200, 150, 100, 50, and 25 µg/ml) were prepared by the proper dilution method. In volumetric flasks, 1 ml of standard solution of each concentration (200, 150, 100, 50, and 25 µg/ml) was taken, and then distilled water (4 ml) was added. In the next step, 5% sodium nitrite ($NaNO_2$) solution (0.3 ml) was mixed to every volumetric flask with shaking and kept for 5 minutes. Then it was mixed with a 0.3 ml solution of 10% $AlCl_3$. 2 ml of a 1 M sodium hydroxide (NaOH) solution was mixed at the sixth minute, and then distilled water is added to adjust the volume to a final amount of 10 ml using distilled water. For each concentration, the UV absorbance was measured at 510 nm against a blank. The absorbance was taken twice, and the mean was used for accuracy.

DPPH free radical scavenging assay

Extracts of each plant material (10 mg) were mixed with 20 ml of ethanol (99-100%) to prepare 500 µg/ml solutions of extract as a stock solution. Another five concentrations of solutions (200, 150, 100, 50, and 25 µg/ml) were prepared. In the same way, various concentrations of ascorbic acid solutions were prepared. A 20 mg DPPH powder was mixed with 500 ml of ethanol (99-100%) to prepare a 0.004% DPPH solution. Then, 2 ml of each concentration's solution was placed in test tubes, along with DPPH solution (6 ml), and each test tube was maintained in the dark for 30 minutes. In the same way, ascorbic acid

solutions were put into seven test tubes, the solution of DPPH was poured, and the tubes were maintained in the dark for 30 minutes. When simply ethanol was used as a blank, DPPH was also administered to the blank test tubes at the same time. A UV spectrophotometer set at 517 nm measured the absorbance of each test tube after 30 minutes. It was noted that the UV spectrometer reading was nullified with a blank solvent, i.e., ethanol, prior to taking absorbance measurements. The readings were noted down carefully. Then the percent inhibition was calculated. The IC_{50} was determined from the percentage inhibition *vs*. conc. graph (Badami et al., 2003).

Anti-inflammatory (Egg albumin) test

Ullah et al. (2014) performed this test with a little change. The 5 ml of reaction mixture contained 2.8 ml of phosphate buffered saline (PBS, pH 6.4), 0.2 ml of fresh hen's egg albumin, and various strengths of extract (2 ml), with final concentrations ranging from 0.12 to 0.36 % (v/v). As a negative control, a comparable volume of distilled water was used. The mixes were then heated at 70 °C for 5 minutes after being incubated at 37 ± 2 °C temperature with the help of a BOD incubator. Using the vehicle as a blank, the absorbance of it was used to measure at 660 nm after cooling. The reference medication, acetyl salicylic acid, was employed at a final concentration of 100 g/ml and was handled in the same way for the purposes of determining absorbance. The proportion of protein denaturation inhibition was estimated using the following formula:

$$\% \text{ Inhibition} = [(\text{Absorbance}_{\text{Control}} - \text{Absorption}_{\text{Test}}) / \text{Absorbance}_{\text{Control}}] \times 100$$

Anti-diarrheal (Castor oil-induced diarrheal) assay

Galvez et al. (1993) and Taufiq and Hossain et al. (2005) devised a systematic approach for studying castor oil-induced diarrhea. Animals (n = 9) were equally separated into three main groups (control, positive control (PC), and test treatment) after fasting them for 24 hours. Individual cage was used to place each mouse, which was lined with absorbent paper on the bottom. 0.1% Tween-80 was used to make aqueous solutions of the extract. The test groups received suspensions of *A. montanum* leaf extract at oral doses of 250 and 500 mg/kg body weight 40 minutes previous to the administration of 0.3 ml of castor oil to each mouse; the control group was given purified water with 0.1% Tween-80; loperamide was administered in the mice of the PC group at 3 mg/kg per body weight. After being administered castor oil, individual animals from each group were kept in separate cages with absorbent paper bottom and monitored for diarrhea every hour for 4 h. During the 4-hour period, the number of feces or any other fluid substance that discolored the adsorbent paper was counted and recorded for each mouse. Each mouse's latent duration was also recorded. Used papers were replaced with new ones at the start of each hour. The total number of feces expelled from the animals was recorded throughout a 4 h observation period.

Anti-pyretic (Brewer's yeast induced fever) test

The antipyretic test was carried out with the help of a method using Brewer's yeast to produce fever (Tomazetti et al., 2005; Turner, 1965). A clinical digital thermometer was used to record each rat's baseline rectal temperature at zero hour. The generation of

pyrexia in mice was produced after inducing a Brewer's yeast solution (20% w/v) in distilled water through subcutaneous injection at a dosage of 20 ml/kg body weight. The rectal temperature of each animal was measured after 18 h following Brewer's yeast injection, and only animals with a temperature increase of at least 1 °F were chosen for the experiment. The total 12 animals were equally divided into four groups randomly. Orally, 1% Tween-80 in normal saline was given in the control group. The conventional medicine paracetamol was orally administered to the PC group at a dosage of 100 mg/kg. Methanol extract was given to the test groups in oral doses of 250 and 500 mg/kg. The temperature of each animals was recorded at 0, 1, 2, 3, and 4 h after the therapy.

Analgesic (Acetic acid-induced writhing) test

This test was carried out with the help of a method defined by the Ahmed et al. (2004) and Ali et al. (2017). Animals were randomly separated into control, PC, and test groups after fasting for 18 hours with water.

Individual cage was used to place each mouse, which was lined with absorbent paper on the bottom. 0.1% Tween-80 was used to make aqueous solutions of the extract. A feeding needle was used to provide the test samples, control and diclofenac sodium orally. The writhing-inducing drug, acetic acid solution (0.7%), was then given to each animal group intraperitoneally. The number of writhing was measured for 15 minutes after a 5-minute gap to allow for acetic acid absorption.

Anxiolytic-like activity test

For a comprehensive evaluation of the anxiolytic-like potential, various protocols, which include swing, open-field, and light-dark tests, were adopted as mentioned below.

Swing test (SWT)

The swing test was adopted, which is a conventional, simple, and straightforward behavioral test that simply entails touching the animal by its tail and documenting the direction of swings it makes over a period of time. The animals were individually placed in a Plexiglas box (40 × 40 × 35.5 cm) and given two minutes to habituate and achieve a neutral stance. The animal was held near the base of its tail, about an inch away. It was then raised to a height of 1 inch above the surface it had been lying on. On either side, the animal was confined in the vertical axis, which was designated as no more than "lo." When the animal moved its head off of the vertical axis to either side, it was recorded as a swing. For the next swing to be tallied, the animal must return to the vertical posture before trying another swing. Only one swing was tallied when the animal swung and redoubled its efforts to migrate toward one side without returning to the upright posture. The animal was then re-suspended and time was reset once it was in a neutral posture. Because swings were frequently exhibited in fewer than one set, the frequency of swings was measured rather than the duration. When the animal was lifted for more than 5 sets, it did not start swinging. The behavior was produced by a mild squeeze on the tail. A hand counter was used to keep track of the swings. To calculate the percentages of left and right swings, the total number of swings on each side was divided by the total number of swings on both sides (Capaldiet al., 1972; Borlonganet al., 1995; Roghaniet al., 2002; Islam et al., 2014).

Open-field test (OFT)

A gray polystyrene box (45 x 45 x 60 cm) was split into two zones: the perimeter and the unpleasant core area of the device. Each mouse was put in the center of the box for the duration of the test, which lasted 5 minutes. The following were the computed standard measures: a) total distance traveled in centimeters; b) as a proportion of total time, time spent in the center zone. The experimental region of the equipment was cleaned with a 0.15% acetic acid solution after each test. Albino mice of either sex (20-25 g) were divided into four groups (Groups I-IV), each with four mice. Prior to the test, they were fasted for the night, although water was provided. Only Group I was kept as a typical control vehicle. Groups III and IV were given various amounts of test extracts, p.o., while Group II was given diazepam (1 mg/kg, i.p.). Each mouse was put in the center of the open field arena 30 minutes after receiving the vehicle, standard, and test extract, and the following parameters were collected during a 10-minute test session (Archer, 1973).

Light–dark test (LDT)

The light-dark test, an insensitive paradigm often employed to measure activity in anxiety disorders, was used. This device comprises of a 40 x 60 x 20 cm acrylic box separated into dark and light chambers. The white colored light chamber (40 x 40 cm) was linked to the dark chamber (40 x 20 cm) by a 7 cm aperture at floor level.

A 60-W white light lamp was suspended in the light chamber above 40 cm high. Animals (n = 12) of 20-25 gm weight of either sex were placed into four groups (Groups I-IV), each with three animals. They fasted for the night before the test, but were given water. The therapy was administered once a day. Each mouse was placed in the light chamber facing the opening into the dark chamber 30 minutes after administration of the vehicle, standard drug, and test extract to different groups, and the following observations were manually recorded during a 10-minute trial: duration of immobility, number of squares crossed, and time spent in the light compartment (Lister, 1990).

Statistical analysis

The data were examined statistically using one-way analysis of variance (ANOVA) across time, followed by Dunnett's post hoc multiple comparison test, using GraphPadPrism software (version: 6.0), with $p < 0.05$ at a 95% confidence interval.

3. Results

Phytochemical screening test

According to preliminary phytochemical research, the plant's methanol extract contains amino acids, fixed oils, flavonoids, glycosides, oleoresins, terpenoid, tannins, steroids, and other compounds (**Table 1.**).

Acute toxicity (A. cepa) test

The largest root growth (RG) inhibition in A. cepa was 4.0 mg/ml after 24 hours, whereas the lowest was 0.5 mg/ml. A reduction in RG was seen in 0.5-1.0 mg/ml extract (CAM, HAM) at 24 h, while in 2.0-3.0 mg/ml extract (MAM) at 24 h. The half-minimal inhibitory concentrations (IC_{50}) calculated for the fractions of MAM, CAM, and HAM are 1.57 ± 0.07, 0.61 ± 0.07, and 1.25 ± 0.06, respectively, and the fraction of MAM, CAM, and HAM has shown an LD_{50} value of 5.13, 3.41, and 4.65 mg/ml, respectively (**Table 2.**).

Table 1. Phytochemical characterizationof the crude methanol extract of *A. montanum*

Phytochemical groups	Test results
Flavonoids	+
Glycosides	+
Tannins	+
Steroids	+
Saponins	+
Oleoresins.	+
Amino acid	+
Fixed Oils	+
Sterols	+

Table 2. Toxic effects of crude extracts and control on *Allium cepa*

Treatments		Root length in cm			% inhibition of root growth			IC_{50} [CI; R^2]
		MAM	CAM	HAM	MAM	CAM	HAM	
VEH		14.54 ± 0.96	19.34 ± 0.10	26.50 ± 0.13	-	-	-	-
Conc. (mg/ml)	0.5	3.21 ± 0.43	1.96 ± 0.22	3.21 ± 0.41	77.92	89.86	87.88	MAM: 1.57 ± 0.07 mg/ml [1.01- 2.43 mg/ml, 0.89] CAM: 0.61 ± 0.07 mg/ml [0.41-0.92 mg/ml; 0.93] HAM: 1.25 ± 0.06 mg/ml [0.86-1.82 mg/ml; 0.92]
	1.0	1.85 ± 0.26	1.44 ± 0.15	1.61 ± 0.27	87.27	92.55	93.92	
	2.0	1.64 ± 0.14	0.61 ± 0.55	1.51 ± 0.15	88.72	96.84	94.30	
	3.0	1.45 ± 0.26	0.41 ± 0.64	1.56 ± 0.27	90.02	97.88	94.11	
	4.0	0.82 ± 0.14	0.41 ± 0.42	0.86 ± 0.11	94.36	97.88	96.75	

Value are Mean ± SD (n = 5); VEH: Vehicle (distilled water); MAM: Methanol extract of *A. montanum* ; CAM: Chloroform extract of *A. montanum*; HAM: n- Hexane extract of *A. montanum*; IC_{50}: Concentration required to inhibit 50% of the root growth; CI: Confidence of interval; R^2: Coefficient of determination

Table 3. DPPH radical scavenging capacity of *A. montanum*

Parameters		% radical scavenge			
		MAM	CAM	HAM	AA
Conc. (µg/ml)	25	12.36 ± 0.01	12.25 ± 0.01	4.52 ± 0.01	40.58 ± 0.01
	50	21.74 ± 0.01	23.95 ± 0.01	13.35 ± 0.01	92.56 ± 0.01
	100	43.55 ± 0.01	36.97 ± 0.01	25.55 ± 0.01	96.10 ± 0.01
	150	60.63 ± 0.01	61.47 ± 0.01	48.45 ± 0.01	96.69 ± 0.01
	200	87.19 ± 0.01	80.13 ± 0.01	70.52 ± 0.01	97.48 ± 0.01
IC_{50} (µg/ml***)***		103 ± 0.05	108.7 ± 0.05	143.1 ± 0.03	27.44 ± 0.01
CI (µg/ml***)***		75.06-141.4	77.58-151.4	115.4-177.6	25.50-29.52
R^2		0.94	0.92	0.95	0.99

Values are Mean ± SEM (n = 3), MAM: Methanol extract of *A. montanum* ; CAM: Chloroform extract of *A. montanum*; HAM: n- Hexane extract of *A. montanum* ; IC_{50}: Half-minimal inhibitory concentration; CI: Confidence of interval; R^2: Coefficient of determination

Antioxidant assay

As mg eq. of quercetin and mg eq. of gallic acid, the plant's total tannin, total flavonoid, total phenolic, and content were estimated. The plant contains 80.3 mg GAE/100 g, 58.66 mg QE/100 mg, and 39.60 mg GAE/100 g of total phenol, total flavonoids, and total tannin, respectively.

The methanol leaf extracts of *A. montanum* (MAM) showed concentration-dependent DPPH radical scavenging capacities. At 200 g/ml, MAM had the highest capability in DPPH scavenging assay. The IC_{50} calculated for MAM, CAM and HAM were 103 ± 0.05, 108.7 ± 0.05, and 143.1 ± 0.03 µg/ml, respectively.

The standard drug, ascorbic acid (AA), also showed a concentration-dependent DPPH radical scavenging capacity. The IC_{50} value of AA was 27.44 ± 0.01 µg/ml (**Table 3.**).

Anti-inflammatory activity

The crude methanol extract of the plant demonstrated an anti-inflammatory activity in an egg albumin (*in vitro*) test in a concentration-dependent manner. At 0.36% (v/v), the extract showed the greatest prevention of albumin denaturation. The test sample had a greater anti-inflammatory impact than the usual ASA at 200 µg/ml. As indicated in **Table 4**, the EC_{50} values for the three fractions of MAM, CAM, and HAM were 89.10 ± 0.07, 92.85 ± 0.0 7 and 117.7 ± 0.0 7 µg/ml, respectively, whereas the normal EC_{50} of ASA was 93.86 ± 0.08 µg/ml. In contrast to ASA, a multiple evaluation of the standards and concentrations for each test reveals that the fractions at 92.85 ± 0.07 and 89.10 ± 0.07 µg/ml had significant impacts (**Table 4.**).

Anti-diarrheal (castor oil-induced diarrheal) test

In this experiment, the extract at a dose of 500 mg/kg per body weight provided a significant effect compared to the standard drug (loperamide), while a 250 mg/kg dose of body weight provided a moderate effect.

Table 4. Anti-inflammatory activitiy of crude extracts and standard drug in egg albumin

Parameters		**The percent inhibition of protein denaturation (%IPD)**			
		MAM	**CAM**	**HAM**	**ASA**
Conc. (µg/ml)	25	22.5 ± 0.03	21.5 ± 0.02	16.6 ± 0.01	22.5 ± 0.01
	50	28.5 ± 0.03	27.5 ± 0.02	21.3 ± 0.01	27.6 ± 0.02
	100	43.5 ± 0.03	42.5 ± 0.02	35.8 ± 0.01	39.7 ± 0.03
	150	66.7 ± 0.03	65.9 ± 0.02	55.7 ± 0.01	62.9 ± 0.01
	200	81.5 ± 0.03	77.6 ± 0.02	67.6 ± 0.01	87.5 ± 0.02
EC_{50} (*µg/ml*)		89.10 ± 0.07	92.85 ± 0.07	117.7 ± 0.07	93.86 ± 0.08
CI (*µg/ml*)		57.25 - 138.7	60.05 - 143.6	77.48 - 178.9	57.45 - 153.4
R^2		0.89	0.89	0.87	0.86

Values are mean ± SEM; MAM: Methanol extract of A.montanum; CAM: Chlorofrom extract of *A. montanum*; HAM: n-Hexane extract of *A. montanum* ; ASA: Acetyl salicylic acid; EC_{50}: Half-maximal inhibitory concentration; CI: Confidence of interval; R^2: Coefficient of determination

Table 5. Diarrheal secretions of mice in different treatment groups at 1st, 2nd, 3rd and 4th hr

Treatment groups	Dose (Route of admin.)	Latency (min)	1st hr	2nd hr	3rd hr	4th hr
NC	10 ml/kg (p.o)	25 ± 0.01	7.58 ± 0.02	4.33 ± 0.02	4.78 ± 0.02	2.09 ± 0.02
LOP	3 mg/kg (p.o.)	75 ± 0.01	3.29 ± 0.02	3.17 ± 0.02	0.00 ± 0.02	0.00 ± 0.00
MAM	250 mg/kg (p.o.)	55 ± 0.01	5.18 ± 0.02	4.23 ± 0.02	3.08 ± 0.02	2.58 ± 0.02
	500 mg/kg (p.o.)	65 ± 0.01	5.09 ± 0.02	3.18 ± 0.02	1.00 ± 0.00	0.00 ± 0.00
Values are mean ± SEM (n = 3); MAM: Methanol leaf extract of *A. montanum*; LOP: Loperamide.						

The crude extract reduced the stool count in both test groups. Four hours after the administration of castor oil, there was no more excretion of diarrheic feces in any of the groups of animals (**Table 5.**).

Anti-pyretic (Brewer's yeast induced fever) test

Table 6 shows the antipyretic activities of several dosages of the test drug (250 and 500 mg/kg), standard (Paracetamol, 100 mg/kg), control, and negative control (NC) in the brewer's yeast produced fever test. When compared to the control, the MAM at concentrations of 500 and 250 mg/kg of body weight had a mild impact. At the 4th hour, the mouse model in group-IV (500 mg/kg) demonstrated a decrease in body temperature.

Analgesic activity (Acetic acid-induced writhing) test

Table 7 shows the impact of the methanolic extract of *A. montanum* leaf on mice in the acetic acid-induced writhing test.

Table 6. Antipyretic effect of *A. montanum* in Brewer's yeast-induced pyrexia in mice

Treatment and dose	Rectal temperature (°F)					
	Basal temp.	0 hr(after 18 hr)	1 hr	2 hr	3 hr	4 hr
Control (10 ml/kg, normal saline)	33.33 ± 0.002	35.12 ± 0.02	35.11 ± 0.01	35.13 ± 0.01	35.15 ± 0.02	35.12 ± 0.01
PARA (100 mg/kg)	33.66 ± 0.01	35.55 ± 0.02	34.65 ± 0.01	33.75 ± 0.01	33.66 ± 0.02	33.66 ± 0.01
MAM (250 mg/kg)	34.89 ± 0.01	35.85 ± 0.02	35.71 ± 0.01	35.64 ± 0.01	35.61 ± 0.02	35.48± 0.01
MAM (500 mg/kg)	34.52 ± 0.01	35.19 ± 0.02	35.13 ± 0.01	35.03 ± 0.01	34.96 ± 0.02	34.27 ± 0.01
Values are mean ± SEM (n = 5); MAM: Methnolic extract of *A. montanum*; PC: Paracetamol (PARA)						

Table 7. Mean writhing and percentage protection in the treatment groups

Treatment groups	Dose (Route of admin.)	Mean of writhing	% writhing	%protection
NC	10 ml/kg (p.o)	36	100	0
Diclofenac-Na	25 mg/kg (p.o.)	6.00	16.66	83.34
MAM	500 mg/kg (p.o.)	6.67	18.22	81.88
	250 mg/kg (p.o.)	13.33	37.02	62.98
Values are mean ± SEM (n = 3); MAM: Methanolic extract of *A.montanum.* PC: Diclofenac-sodium				

Table 8. Anxiolytic-*like* effects of crude extracts and controls in *Swiss* mice

Treatment and dose	OFT			HCT	SWT	LDT
	Number of Field Cross	Number of Grooming	Number of Rearing	Number of Hole Cross	Number of swings	Residence in dark (Sec)
Control (10 ml/kg, p.o.)	145 ± 0.01	46 ± 0.02	48 ± 0.01	25 ± 0.02	17 ± 0.01	125 ± 0.02
DZP (2 mg/kg, i.p.)	111 ± 0.01	19 ± 0.02	15 ± 0.01	12 ± 0.02	10 ± 0.01	155 ± 0.02
MAM (250 mg/kg, p.o.)	109 ± 0.01	33 ± 0.02	23 ± 0.01	15 ± 0.02	10 ± 0.01	132 ± 0.02
MAM (500 mg/kg, p.o.)	98 ± 0.01	18±0.02	13 ± 0.01	11 ±0.02	06 ± 0.01	145 ± 0.02
Values are mean ± SEM (n = 3); DZP: Diazepam; LDT: Light-dark test; HCT: Hole cross test; MAM: Methanol extract of *A. montanum*; OFT: Open field test; SWT: Swing test.						

The crude extract generated the writhing inhibition (81.88%) in test animals at a dosage of 500 mg/kg of body weight, whereas the crude extract at a dose of 250 mg/kg of body weight produced 62.98% inhibition of writhing. When compared to the negative control, the findings were statistically significant ($p < 0.005$).

Anxiolytic-like activity test

SWT

The number of swings in the test groups (Gourp-III and IV) (250 and 500 mg/kg, p.o.) mouse model was lower than in the control group in the swing test. The outcome was statistically significant ($p < 0.05$) and equivalent to the reference medication diazepam (2 mg/kg).

OFT

The number of squares occupied of each mouse in the open field test was dramatically reduced in the test groups (Gourp-III and IV) throughout the research period. The extract had stronger CNS depressant action than the conventional medication, and the difference was statistically substantial.

HCT

MAM (250 and 500 mg/kg, p.o.) reduces locomotion in selected mice by lowering the number of holes crossed comparing to the control group in the hole cross test (**Table 8.**). The outcome was statistically significant ($p < 0.05$) and equivalent to the reference medication diazepam (2 mg/kg).

LDT

MAM significantly increased the dark present rate in the test groups (groups III and IV) comparing control group.

4. Discussion

The project work presented here was done to analysis some pharmacological and phytochemical features of the mangrove plant, *A. montanum*. Several portions of this plant are traditionally applied in the treatment of measles, chickenpox, malaria, headaches, stomach aches, ulcers, lumbar pains, eye diseases, relieving chest pain, headaches, and thrush in children, for diuretic and kidney stone removal, and for anti-dermatitis and skin disease curing effects.

This work was based on phytochemical and pharmacological assessment to investigate the metabolites and bioactivity of the plants.

The plants were subjected to drying under the shade, followed by pulverization. After proper grinding and cold extraction with methanol (99-100%), the extraction afforded an 8.00% yield. To obtain a sense of the active

ingredients in the extract, phytochemical assessments revealed the attendance of chemical constituents which include amino acids, fixed oils, flavonoids, glycosides, oleoresin, terpenoids, tannins, steroids, saponins, and sterols.

The total phenolic, total flavonoid, and total tannin content of the plant were calculated as mg eq. of gallic acid and mg eq. of quercetin, and the plant contains 80.3 mg GAE/100g, 58.66 mg QE/100 mg, and 39.60 mg GAE/100 g of total phenol, total flavonoids, and total tannin, respectively. The leaf extract of *A. montanum* showed moderate DPPH radical scavenging in comparison with ascorbic acid. Here, the IC_{50} of three fractions, MAM, CAM, and HAM, was 103 ± 0.05, 108.7 ± 0.05, and 143.1 ± 0.03 µg/ml, respectively. But in overall observation, it has been decided that segments of plant extract MAM and CAM showed moderate antioxidant activity while the HAM showed mild activity.

Furthermore, flavonoids (Pietta, 2000), glycosides (Lee et al., 2005; Katsube et al., 2006), tannins (Zhang and Lin, 2008), terpenoid (Grassmann, 2005), and saponin (Guelcin et al., 2004) exhibit antioxidant activity by inducing enzymatic antioxidant activity and itself as a non-enzymatic antioxidant and scavenging free radicals. From phytochemical screening of *A. montanum*, this plant contains terpenoids, glycosides, flavonoids, tannins, saponins, so the antioxidant activity was inhibited because of these phytochemicals.

In this study, *A. montanum* was found to exhibit a significant role in anti-inflammatory activity. In the egg albumin (*in vitro*) test, the crude extract of the plant exhibited the highest inhibition of albumin denaturation at 200 µg/ml with fractions of MAM and CAM compared to the same standard (ASA) concentration. The extract at 200 µg/ml showed significant anti-inflammatory capacity because of the chemical constituents, which included flavonoids, glycosides, terpenoids, tannins, saponins, and steroids (Takagi et al., 1980; Dıazet al., 2004; Loke et al., 2008; Liang et al., 2010).

In the castor oil-induced antidiarrheal test, *A. montanum* exhibited substantial ($p < 0.005$) effects. The findings of this study demonstrated that the MAM showed significant anti-diarrheal activity. The extract's phytochemical investigation confirmed the presence of a variety of bioactive compounds. Phytosterols and flavonoids are among the secondary metabolites discovered to alter the synthesis of lipooxygenase (LOX), cyclooxygenase 1 and 2 (COX-1, COX-2) and hence decreasing the production of prostaglandin (Awad et al., 2005). The extract at 500 mg/kg dose provides a significant consequence on the reference drug loperamide, while a 250 mg/kg dose provides a moderate effect. The inclusion of phytochemicals such as phytosterols, flavonoids, saponins, and tannins, which act individually or synergistically, may be responsible for the extract's antidiarrheal properties.

A. montanum includes flavonoids, according to phytochemical study. Flavonoids are polyphenols that may be found in various amounts in almost all plants. Flavonoids have also been discovered to block the transcription and formation of prostaglandin synthase (COX-2) (O'Leary et al., 2004; Hamalainen et al., 2011). As a consequence, the existence of flavonoids might explain *A. montanum*'s antipyretic activity. With increasing concentrations of *A. montanum* extract, the efficacy of the antipyretic action was seen to rise. This can be attributed to a higher concentration of the extract component that has antipyretic properties. The results of this investigation show that MAM have mild antipyretic effects at dosages of 250 and 500 mg/kg of body weight, and that suppression of inflammatory mediator production and/or

release may be the principal mechanism of action. The antipyretic properties of MAM justify its use by traditional medicine practitioners in the treatment of fever.

The approach of testing peripherally active analgesics using an acetic acid-induced writhing model is helpful due to the fact that it focuses on assessing the medication-induced reduced number of pain-inducing paramers in the test animals. Acetic acid, used in this model, works by inducing prostaglandin production in animals, which can be inhibited by the test sample through peripheral mechanisms (Ferdous et al., 2008).

The presence of analgesic principles operating through the prostaglandin pathways might explain MAM's considerable pain reduction. The presence of bioactive chemicals is thought to be responsible for the reported analgesic effect. Flavonoids are thought to play a function in analgesic action through interacting with prostaglandins (Rao et al., 2008; Vanu et al., 2006).

Extracts at doses of 250 and 500 mg/kg inhibited the writhing reflex by 62.98 and 81.88%, respectively, in an analgesic activity test using the acetic acid induced writhing method, whereas the standard drug diclofenac-Na inhibited the writhing reflex by 83.34% at a dose of 25 mg/kg body weight.

The current investigation found that an oral administration of a MAM to mice generated a strong anxiolytic effect in four well-established anxiety experimental systems (e.g., HCT, OFT, LDT, and SWT). Anxiety-related behaviors in mice were dramatically reduced in various animal models, demonstrating that extract administration reduced anxiety in mice. Flavonoids, phenolic compounds, tannins, saponins, steroids, glycosides, and alkaloids were discovered during the phytochemical screening of EEAPR. It's probable that these compounds are involved in the title plant's anxiolytic activity.

Conclusions

The leaf extract of *A. montanum* demonstrated the existence of flavonoids, terpenoids, steroids, saponins, tannins, and oleoresin. The extract exerted a concentration-dependent toxic effect on *A. cepa*. The extract inhibited egg albumin denaturation depending on concentration. The extract exhibited significant antioxidant capacity. The MAM showed anti-diarrheal, anti-inflammatory, and analgesic effects. In a dose-dependent way, the MAM also showed antipyretic and anxiolytic properties in *Swiss* mice. It's worth noting that *A. montanum* might be a source of phytotherapeutic lead compounds. Further studies are necessary to isolate and investigate the responsible bioactive substances of this hopeful medicinal herb.

Conflict of interest

None declared.

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Authors` contributions

F. Zaman- laboratory studies, data collection, preliminary drafting; R. Hossain- laboratory studies, data collection; S. Molla, A.K. Das and M.H. Bappi- laboratory studies, data collection; M.T. Islam- data manipulation, data analysis, final drafting; C. Sarkar and O. Atolani - final drafting; R. Rouf- work design, data manipulation, data analysis, final drafting.

Consent for publication

The authors declare their consent for publication.

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READING SYMBOLS IN JAPANESE GARDEN

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Abstract: Creating a Japanese garden raises a number of methodological questions. The form of these gardens are well known, but their religious symbols are not well researched in English literature. The aim of the research is to introduce and interpret the religious symbols and references present in Japanese gardens, to categorize their appearance by taking into account their unique characteristics, their role in the garden, contributing to a deeper understanding and the understanding of the form-shaping approach. The gardens presented by this study are closely related to the practice of the religious community that creates or maintains them. The garden elements do not only serve an aesthetic purpose, but with their spatial presence they constantly refer to religious teachings or attitudes for those who are able to read them.

Keywords: art, buddhism, forms, purpose, sacrality

1. Introduction

There is a growing interest in Japanese culture, including horticulture. It is the works of the British architect, Josiah Conder, from the turn of the 20th century where we have found the first detailed descriptions about the design and rules of Japanese gardens. Information on the subject is available through a number of channels and is becoming increasingly accessible. During our research, we managed to find and classify nearly five hundred English-language publications (books and journal articles) dealing with the history and art of Japanese gardens. Most of the publications contain descriptions and illustrations of the formal elements, without exploring the religious messages and philosophies that influence horticulture. The intellectual content important for the rich and artistic image and forms of the Japanese garden are therefore pushed into the background. The aim of the research is to introduce and interpret the religious symbols and references present in Japanese gardens, to categorize their appearance by taking into account their unique characteristics, their role in the garden, contributing to a deeper understanding and the understanding of the form-shaping approach.

About the literature

The first English-language book that, beyond the general description of the island country, also mentions Japanese garden as the surrounding of buildings is from Edward S. Morse who published his richly illustrated book titled "Japanese Homes and Their Surroundings" in 1885, complementing the work of J.J. Rein on Japan (Rein, 1883). The book describes the Japanese house, its types, the layout of the interior, the entrances and the access to them, the garden, and other related topics. The section on the garden includes 23 pages on specific garden elements such as stone monoliths, stone lanterns, bridges, summer pavilions, ponds, paths, dwarf trees, flowers, plants. The book also includes illustrated descriptions of a few private gardens.

Morse did not discuss the principles behind the art, which were unknown for him, but he acknowledged their positive impacts in art. According to his description, Japanese people are masters of creating artworks in a simple manner.

The British architect Josiah Conder arrived to Japan in 1877, and soon became a professor at the Imperial Technical University. His first book titled "The Flowers of Japan and the Art of Floral Arrangement" was published in 1892. A year later an exceptionally richly illustrated book of his was published titled "Landscape Gardening in Japan", dealing with the history and principles in Japanese garden art. The source of his work was the book "Tsukiyama teizōden" from Akisato Ritō, written in 1829 on the subject of garden construction. Conder's book focused on the artistic composition of the garden, with some of the philosophical aspects also mentioned briefly in the Introduction, for instance: "The ideal Japanese garden serves primarily as the site of retreat for solitary relief and meditation" (Conder, 1893). Conder referred to the form as an artistic expression of the philosphical background, but did not discuss its Western interpretation, only highlighted its mystique and sacredness.

Information on Japanese gardens are increasingly accessible through various media today. With our research, we managed to find and classify almost 500 English-language publications, books and journals on the history and art of Japanese gardens.

We can conclude that most of the publications deal with the description of compositional elements of Japanese gardens, without a deeper exploration of the spiritual messages and philosophies that influence the garden art.

Displaying the aggregate number according to the year of publication **(Fig. 1)**, we can observe a linear increase from Conder to circa the 1950s. The books on Japanese gardens exploded in number afterwards, while the number of journal articles started to grow rapidly from the 1960s, 1970s. The exponential growth after the Second World War is remarkable, the analysis of the reasons could be the topic of another specific research.

2. Materials and methods

Our research was based on publications which do not only describe the design of garden elements, but also look at the content beyond the form. Most of the literature does not fully explore the approach that determines the form, since the primary goal of the author was to provide a simple and easy-to-understand introduction of the garden scenery and the symbols. Thus, in addition to the publications on Japanese gardens, we also introduce the doctrines of the related religions, which provide the keys for understanding the garden scenery. The introduction of spiritual aspects of the elements of monastery and tea gardens covered by the research also reveals details of their history and everyday function.

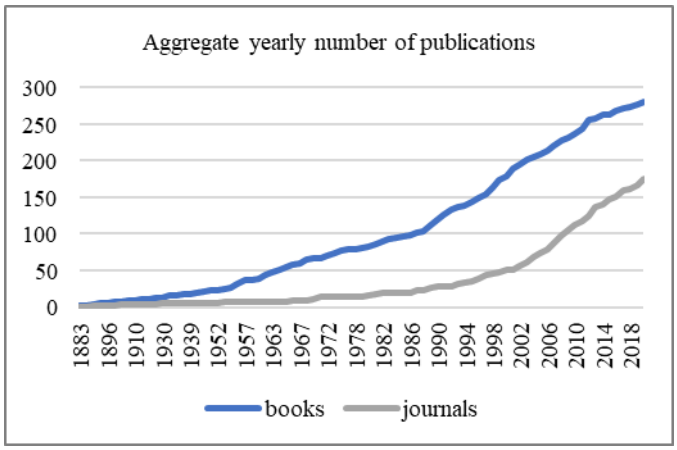

Fig. 1. English-language publications on Japanese gardens (Author, 2018)

3. Results and discussion

Shintoism

In Shintoism, a purged place where a god or spirit (kami) resides is named garden (niwa). The most characteristic feature of these gardens is the rock (iwakura) (Goto and Takahiro, 2016), which is compelling and prominent in the view. This garden element is also worshipped as yorishiro, which refers to the fact that it is a place where a kami resides. And the role of the rope (shimenawa) is to mark the place, the garden element that is worshipped as yorishiro (Keane, 1996).

The rocks are usually replicas of original rock formations existing in nature. Nevertheless, often the rock formations also refer to the belief system of a specific religion.

Buddhist cosmology

Situated in the centre of metaphysical and spiritual universe, Sumeru (Mount Meru), that is shumisen in Japanese, plays an important role in Hindu and Buddhist cosmology. The top of the mountain is the symbol of heaven. This centre is surrounded by a ring of eight lower mountains with eight oceans in between. Man resides in the outer range of mountains situated at the eighth oceans (Goto and Takahiro, 2016). This image also appears in Japanese gardens in a similar way, as a central rock surrounded by several lower rocks. The Buddhist temples and stupas are also symbolic representions of this mountain (Pressing, 2007). The first Buddhist symbol in Japanese gardens was also representing this mountain (Berthier, 1989).

The image of Pure Land

An approach similar to the one related to Meru Hill appears in the so called Pure Land stream of Buddhism. According to their doctrine, there is a place where Amitabha Buddha resides, and where enlightened Buddhist practitioners get to after their death, breaking the endless cycle of rebirth (Skilton, 1997). This place had inspired garden designers, and some of the gardens created in the Heian Period reflected this idea. An island is floating in the foggy ocean, accessible via a bridge, as a reference to the fact that accessing the island (the Pure Land) is possible with the help of Amitabha Buddha (Keane, 1996).

Metapshysical goals using mandalas

The Shingon school of Buddhism was a mystic school in Heian Period Japan. It had more or less united the concept of Buddha with the Shintoist kami. It had happened that a Shinto shrine had been installed in the area of a Buddhist monastery. They considered the hills in a similar way as well as the rocks where,

according to Shinto, a kami resides. These are places where enormous power is concentrated. During their practice, even the hard conditions of their retreats into the hills did not prevent them to partake of this power (Yamasaki et al., 1988). In their practice they used mudras, mandalas and mantras, which had influenced the architecture of the temples and the composition of the religious spaces. To some extent, even the location of the sculptures of Buddha and the pagodas had referred to mandala. Why the use of mandala was so important for this school? The mandala **(Fig. 2)** is not merely a kind of depiction of the belief system of the school, but as they believed the image had also had a sort of mystical power that was possible to invoke by a ritual motion of the hands or by chanting a mantra. The architecture of Shingon monasteries also relates to this, although the layout of the buildings is not considered as a mandala in the classical sense, but it serves the same metaphysical goal that we can see in the case of the mandala (Keane, 1996).

Fig. 2. Womb realm mandala, Shingon tantric school, 9th century (Ismoon, 2019)

Emotions and religious experience

Art had been present in gardens also in the form of poetry (e.g. poetry competitions held in Buddhist monastery gardens). In poetry, the use of "dependent words" (kakekotoba) was typical, expressing double meaning by the means of homophone sounding of specific garden elements. One of the meanings expressed an emotion, while the other referred to a natural feature or phenomenon. For example the word matsu means pine tree as a noun and wait as a verb or kaeru refers both to homecoming and frog (Hirano, 2014). The notion of homecoming represented by the frog is also present in Shinto, as a metaphor expression of the fact that in the course of everyday life man is not in the status of "home".

The haiku below is one of the renown works of the 17th century poet, Matsuo Bashō:

"The old pond —
a frog jumps in,
sound of water."

Buddhist monks were also inspired by the natural scenes of gardens to write poems, which were the descriptions of a specific spiritual experience, as simple and concise

expressions of a flash-like revelation in lyric poetry.

As a Buddhist monk, Musō Soseki was not only a master, but also a garden designer and poet of the period (Soseki et al., 2013):

"In this small hut
are worlds beyond number
Living here alone
I have endless company
Already I have
attained the essence
How could I dare
to want something higher"

The art of Zen Buddhism

The advance of Zen Buddhism to Japan resulted in a novel garden scenery, the source of which originates from the Chinese landscape painting. It is mostly enclosed spaces, with austere simple forms, which support introversion and immersion. The goal of the practice in Zen Buddhism is to reveal the real nature of consciousness behind the superficial secular life, which, although hidden, continuously exists before our very eyes.

The Zen doctrine despises shallowness and excessive decoration **(Fig. 3)**. Beyond the puritan, simple forms of the monastery gardens, this attitude is also conspicuous in various forms of art related to the stream (sumie painting, ikebana flower decorations, tea ceremony, shakuhachi flute, haiku poetry).

Fig. 3. Splashed-ink landscape, 1495, Sesshū Tōyō (Lippit, 2012)

Regarding Zen arts, two phrases are related to the above written. As a word of Chinese origin, yūgen means a sort of mysterious depth. This also means that something exists that is so deeply inside that we can not see it. In Zen practice this refers to the real nature of consciousness. And yohaku no bi means the beauty of empty space, which may also be observed through the subtle representation of the mysterious, dim, invisible spaces in ink painting (sumie) (Keane, 1996). We can relate two Buddhist phrases to these artistic concepts. First, suññatā that is usually translated as emptiness. This emptiness may refer to the objective nature of matters and of course of events, that none of them exist on their own, independent of the other. But may also refer to the opportunity that we have for filling the emptiness. The other phrase, kenshō jōbutsu, is the forth of the four principles of Zen. Kenshō means the revelation of our own nature, by which man transforms to buddha (jōbutsu).

These two examples demonstrate that the artistic phrases expressed the subject matter of the Zen practice, while artistic forms and artworks reflected the spiritual attitudes.

Revealing attitude in karesansui gardens

The karesansui gardens of the Zen Buddhist monasteries are literally dry landscape gardens. According to sakuteiki (Takei and Keane, 2008) this is a place where neither lakes nor ponds exist. The objective was to create a view that is possible to observe looking out from the central building in the garden. The view referred to the compositional principles known also from Chinese painting. Chinese landscape paintings depicting hills often showed the wise hermit in the broad context of the natural world. This broad natural scenery was relocated into the tight enclosed space of the monasteries.

We cannot emphasise enough that the image of a monk sitting in a dry garden for the purpose of meditation is false. According to the monastery rules, in traditional monasteries the place of meditation for the monks is the meditation room (zendō), while other exercises may be carried out anywhere else including the garden. The garden is not a subject of meditation, the Zen meditation is formally determinated, practitioners must look downwards in an angle of 45 degrees, and not to observe the view in front of them. Nevertheless several activities may take place in the garden, such as maintenance, raking, cleaning. These may evoke a meditative state of mind. Regarding the scenery, it is important that water does not appear in dry landscape gardens, but the arrangement of the stones may refer to it **(Fig. 4)**.

That is how the riverbed, that is ordinarily hidden by the turbulent flow of water, appears in the view. Here we would highlight the moment of becoming visible, which is a strong allusion to the subject matters of the previously mentioned yūgen and kenshō. Another instance of composition, the dragon gate waterfall, represents a similar allusion to the religious practice. The story about the denomination of the waterfall (Mansfield and Richie, 2012) highlights a human attitude that is important from the aspect of monastic practice, effort and steadiness.

Fig. 4. A mountain, waterfall, and gravel river at Daisen-in (Ivanoff, 2004)

For the outsider, these types of sights in the monastery garden provide an aesthetic experience, while for the monks they are suitable to evoke and sustain attitudes in relation to their religious practice.

The burning house and the tea garden

According to Musō Soseki, the observation of nature alone is also suitable to evoke the kenshō (Goto and Takahiro, 2016). In his opinion, the image of the natural scenery is the mirror of Buddha-nature, that is to say, through the Zen practice it is possible to perceive our own nature simply by observation.

Another type of gardens that is closely related to Buddhism is the Japanese tea garden (roji). The name referred to the path leading to the tea house located in the rear part of the garden, and was later related to a Buddhist doctrine. The character that means garden appeared in the 17th century in the Lotus sutra (NBMR, 2021), in the parable of the house on fire. In brief, in this parable is a house on fire where children play inside obliviously. Their father tries to warn them but they do not notice it, so that he is not able to get them out of the house. The father then comes to the idea of offering presents provided they come out. This attracts the attention of the children who come out of the burning house to a particular area. The area they arrive to is expressed by these characters. The tea garden is usually translated as dewy earth, but as we can see at the first character it also has a meaning that refers to showing or revealing something.

露 – dew; to show, to reveal

地 – earth; ground, field

If we consider the garden with regard to the parable of the house on fire, then the city, the outer world represents the burning house, while the tea garden the area where we arrive to leaving the burning house behind. The garden is sectioned into several units, the outer garden, the inner garden and the tea house. Arriving from the rush of the mundane world, we can walk down this route. Regarding the parable it is important to note that the story does not say that the children take notice of the burning house when leaving it, it only explains that with the use of presents, which we may also call as smart tools, they are not inside the burning house anymore. The questions of what happens in that area and how does it contrast the image of the burning house, may perhaps be answered through the practices and activities in the garden. The Buddhist attitude appears here so that the directed steps that we make here serve for the purpose of calming down. We can not walk down the garden in any way we want, we can not rush, since there are several places to pause, where we can observe the view of the natural scenery.

It is the contemplation itself what suggests that we are supposed to discover something in this space, something that we did not take notice of yet. The space, from this aspect, is an opportunity. Although we are not in the burning house anymore, yet we can not see this. The presents in the story, the smart tools, may be paralleled with the activity that takes place in the tea garden, that is having a tea, so that the tea is

the present itself, that beckons into this space from the burning house.

Conclusions

In the beginning, Japanese gardens related to religions served to represent the specific belief system, rocks, islands, bridges in the garden were simply tools of pictorial description. Applying the rules and regimes of the belief system to the garden composition, mystic schools wanted to use the garden as a tool in achieving the religious objective then. This is where we can observe a change in the function of the garden scenery.

With the advance of Zen, the truth behind the scenery appeared in the gardens analysed. The facilitation of a certain human attitude (calming down, effort, steadiness) with the deliberate use and composition of garden elements is related to this search for the truth, and serves the religious practice.

Besides, the space of the garden may also be considered as an opportunity, since the tea garden provides an opportunity for discovering something by leaving the rushing world behind. And, according to Soseki, the contemplation of the garden scenery is an activity equal to meditation, since the natural scenery observed is a clear reflection of the Buddha-nature.

Regarding composition, the question is what role the garden scenery might play if the view is not related to some mental practice. The scenery that we can observe in monastery gardens is possible to relate to the compositional principles of Chinese landscape painting. However, for us who are not living in a monastery, and are not related to the practice of the specific religion, the garden scenery does not become animated, and remains no more than a painting with a related story.

Table 1. Religious purposes of garden images

Religion	**Garden image**	**Religious purpose**
Shintoism	Represent nature	to describe the belief system
Buddhism	Represent buddhist cosmology	to describe the belief system
Pure Land Buddhism	Pictorial description of celestial realm	to emphasize the possibility to get there
Shingon School of Buddhism	Using mandala images	to revoke mystical power during the practice
Shintoism and Buddhism	Homophone sounding of garden elements	to describe emotions or religious experience
Zen Buddhism	Dry landscape garden	to evoke and sustain religious attitudes
Zen Buddhism	Tea garden represents mountain pathway	to leave the burning house behind

Conflict of interest

None declared.

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THE ROLE OF NOREPINEPHRINE IN THE REGULATION OF GROWTH, ADHESION AND INVASION OF *PSEUDOMONAS AERUGINOSA* IN HUMAN LUNG CARCINOMA CELL CULTURE

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Abstract: Mammalian hormones are shown to affect not only regulation of mammalian homeostasis but also play roles in cross-talk between microorganisms and their hosts. In our study, the roles of norepinephrine (NE) at two different concentrations on growth alterations, adhesion - invasion of *Pseudomonas aeruginosa* were investigated.

The effects of NE on the growth, adhesion - invasion of *P. aeruginosa* ATTC 27853 were examined in human lung carcinoma (A549) cell culture. We analysed two concentrations (**HNE:** 0.04µg/mL and **LNE**: 0.0017 µg/mL) of NE considering NE's psychological levels in a healthy individual to imitate *in vivo* conditions of the host. Bacterial counts of growth and adhesion-invasion were examined by the colony counting method. Growth of bacterium was significantly reduced in the presence of NE at 90' incubation (p: 0.0004 for high concentration and p: 0.0003 for low concentration); on the other hand, at 180' minutes incubation, only low concentration reduced the growth ($p<0.0001$). While adhesion was increased in the presence of low NE concentration (p: 0.013), there was no alteration in invasion of *P. aeruginosa* in the presence of NE ($p>0.05$). Our results suggested that NE has different effects on the growth and adhesion of *P. aeruginosa* and these effects depend on concentration and incubation period.

Keywords: *Pseudomonas aeruginosa*, norepinephrine, growth, adhesion, invasion, A549 cell culture

1. Introduction

It is known that when a microorganism reaches the host tissues, host conditions including hormones have become the microbe's environment. Microbial endocrinology is a research area that aims to determine the roles of mammalian hormones on microbial behaviors during the infection process. Previous studies suggested that different kinds of hormones have effects on the modulation of growth, virulence properties, antimicrobial susceptibilities and gene expressions (Plotkin and Viselli, 2000; Lyte and Freestone, 2010; Lyte and Cryan, 2014; Fteita et al., 2014; Plotkin and Konakieva, 2017; Lyte et al., 2021).

It seems that microorganisms sense and respond to host hormones. Consisting with this opinion, some authors focused on stress hormones and their metabolic activities. The

ability of hormones to stimulate the growth and the pathogenicity of microorganisms have been shown (Bearson, 2016; Cambronel et al, 2020; Freestone et al., 2007; Gümüş et al., 2019; Lyte et al., 2003; Truccollo et al., 2020). On the other hand, in the intensive care units, it is well known that treatment of patients with catecholamines, especially norepinephrine and dopamine, as inotropic drugs, can be helpful to overcome infectious stages.

Pseudomonas aeruginosa is an opportunistic pathogen and it is responsible for keratitis, cystic fibrosis, ulcers, wound - surgical site infections. Moreover, the bacterium is a common hospital-acquired agent and most strains are resistant to three or more antibiotics including cephalosporins and carbapenems used to treat ordinary *Pseudomonas* infections (Driscoll et al., 2007; Moore and Flaws, 2011).

Therefore, in this study, the role of norepinephrine (NE) in the regulation of growth, adhesion and invasion properties of *Pseudomonas aeruginosa* was examined in human lung cell carcinoma cell (A549) culture as an infection model.

2. Materials and methods

Bacterium, medium and hormone

In the present study, *Pseudomonas aeruginosa* ATCC 27853 strain was used. The bacterial suspension (approximately 10^7 CFU/mL) was prepeared in Mueller Hinton broth (MHB) to provide overnight culture.

The effects of norepinephrine (NE) (Sigma) at two concentrations, **low concentration:** 0.0017 and **high concentration:** 0.04µg/mL, were chosen to mimic *in vivo* conditions of the host by considering their psychological levels in a healthy individual.

Human lung carcinoma cell line (A549)

The human lung carcinoma cell line (A549) was used for growth, adhesion and invasion assays. Fetal bovine serum (FBS) (10%) (Biowest, S1810-500), 2mM L-glutamine (1%) (Biological Industries, BI03-020-1B) and penicillin/streptomycin (1%) (Biological Industries, 03-031-1B) added into Dulbecco's modified Eagle medium (DMEM) (Sigma, 5546) was used to prepeare A549 cell culture.

A549 cells were cultured in 96 and 24-well culture dishes for bacterial growth, adhesion and invasion experiments, respectively. In order to maintain a confluent monolayer cell culture, seeding density was arranged to almost 5×10^4 cells for 24-well and 1×10^4 cells for 96-well culture dishes, then the plates were incubated at 37 °C for 24 hours under 5% CO_2 conditions.

Inoculation of cell culture (A549)

A549 cells were inoculated with overnight cultures of *P. aeruginosa* strain. Prior to inoculation of strain, medium was aspirated and replaced with antimicrobial solution-free DMEM. Then, the two concentrations of NE were added to each well (20µL for each well/96-well plate and 50µL for each well/24-well plate) for experiments; as controls, cell cultures without hormones were used, for each experiment. The culture dishes were incubated for one hour, at 37°C before experiments, after incubation, A549 cells were inoculated with approximately 10^7 CFU/mL *P. aeruginosa*. Then, plates were incubated at 37°C for different periods according to experiments.

Growth assay

Growth alterations were determined by colony counting method. Cells were inoculated with *P. aeruginosa* strain as mentioned above and incubated for 90 and 180 minutes. After incubation, 20µl cell culture suspensions from

each well (with and without hormone at two concentrations) were inoculated on Mueller Hinton agar for comparison of colony numbers. All experiments were independently repeated three times and all conditions were examined thrice.

Adhesion and invasion assays

The effect of NE on adhesion and invasion of bacteria was performed as reported previously (Artini et al., 2011; Castillo et al., 2017). Inoculated A549 cells with bacteria were incubated at 37°C for one hour. After incubation, to remove unbound bacteria, cells in wells were washed three times with phosphate buffer saline (PBS). For lysing cells, 500µl 0.025% Triton X-100 was added to the wells and the plates were incubated for 5 minutes at 37°C under 5% CO_2 conditions. Lysates were homogenized and inoculated on Tryptic Soy agar (TSA) and incubated for 24 hours at 37°C. The real number of adhered bacteria was detected by colony counting (CFU).

For the detection of invasive bacterial numbers, bacteria inoculated-A549 cells were incubated at 37°C, for three hours, then, PBS was used three times for washing inoculated cells and a fresh medium supplemented with gentamycin (200µg/mL) was used for killing extracellular bacteria. After this stage, the plates were incubated for one hour, at 37°C. A549 cells were lysed with Triton X-100 and for quantification of invasive bacteria, homogenized cell lysates were inoculated as mentioned above.

Both determination of adhesive and invasive bacteria (as CFU/mL) obtained from cell lysates of inoculated cell cultures with/without NE (both two concentrations) were compared. All conditions were repeated three times and each experiment was carried out thrice.

Statistical analysis

Differences between results of experimental - and control conditions were statistically analyzed. The analyses were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test for growth alterations. Two-way ANOVA followed by Dunnett's multiple comparisons test was used for the analysis of adhesion and invasion results. All results were presented as mean ± SD. Differences with p values less than 0.05 were considered significant.

3. Results and discussion

Results

The alteration of bacterial growth in the presence of norepinephrine

Both two concentrations of NE were statistically significantly decreased the growth of *P. aeruginosa* strain after 90 minutes incubation (p:0.0004 for HNE and p:0.0003 for LNE) **(Fig. 1)**. On the other hand, when incubation was prolonged to 180 minutes, the growth was only statistically significantly reduced in the presence of low NE concentration ($p<0.0001$).

The alteration of bacterial adhesion and invasion in the presence of norepinephrine

Low NE was shown to be significantly enhanced the adhesion of *P. aeruginosa* strain (p: 0.013). However, the presence of NE has no effect on invasion of *P. aeruginosa* ($p>0.05$) **(Fig. 2)**.

Discussion

Over the years, numerous studies have shown that mammalian hormones have roles not only in the regulation of homeostasis in mammalian hosts but also in the regulation of microbes' behaviors. With the long coexistence of the microbes and their hosts, microbes had to adapt to host conditions.

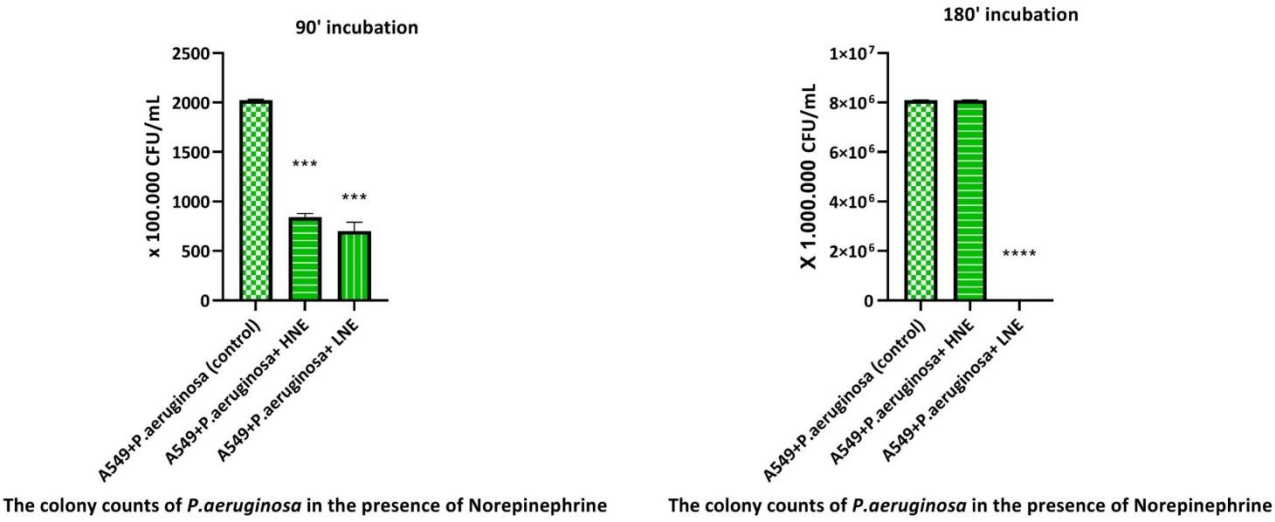

Fig. 1. The alteration of bacterial growth in the presence of NE

The growths of *P. aeruginosa* in A549 cell culture with-without NE were carried out using one-way ANOVA followed by Dunnett's multiple comparisons tests.

HNE: High concentration of norepinephrine (0.04 µg/mL), **LNE:** Low concentration of norepinephrine (0.0017 µg/mL) *****, ******: Significant at p: 0.0004, p: 0.0003 and p<0.0001 levels, respectively

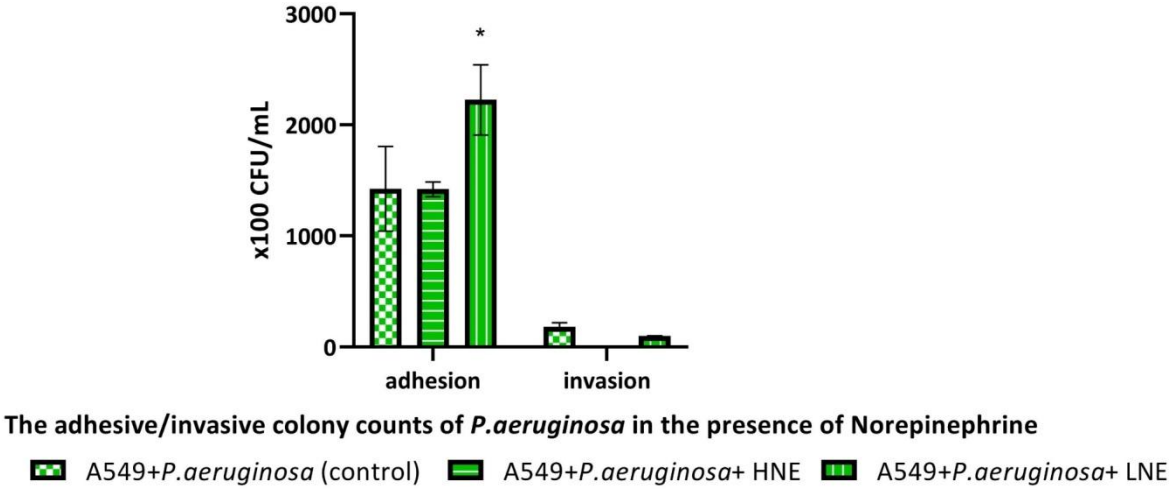

Fig. 2. The alteration of bacterial adhesion and invasion in the presence of NE

The adhesion and invasion of *P. aeruginosa* to A549 cell culture with-without NE were examined using two-way ANOVA followed by Dunnett's multiple comparisons tests.

HNE: High concentration of norepinephrine (0.04 µg/mL), **LNE**: Low concentration of norepinephrine (0.0017 µg/mL) ***:** Significant at p: 0.013 level

As evolution requires, microorganisms modulate their growth, metabolism, virulence, susceptibilities and gene expressions. Since Lyte and Ernst proposed microbial endocrinology concept as a new approach to understanding the infectious process, many researchers reported that mammalian hormones provide a bi-directional interaction between host and microorganism via inter-kingdom signaling (Kornman and Loesche, 1982; Lyte and Ernst, 1992; Plotkin and Viselli, 2000; Lyte et al., 2003; Plotkin and Konakieva, 2017; Gonçalves et al., 2020; Truccolloet al., 2020).

Based on this approach, the present study was investigated the role of norepinephrine, a catecholamine, in the regulation of growth, adhesion and invasion of *Pseudomonas aeruginosa* strain in human lung carcinoma cells imitating host conditions as much as possible.

In previous studies, it has been shown that norepinephrine affected the growth of various microorganisms such as *Campylobacter jejuni, Escherichia coli, Prevotella species, Porphyromonas gingivalis, Streptococcus mutans, Streptococcus pneumoniae, Staphylococcus epidermidis, Vibrio harveyi, Helicobacter pylori, Aeromonas hydrophila and Pseudomonas aeruginosa* (Lyte et al., 2003; Doherty et al., 2009; Gonzales et al., 2013; Sandrini et al., 2014; Yang et al., 2014; Xu et al., 2015; Boyanova, 2017; Gao et al., 2019; Gümüş et al., 2019). While, most microorganisms' growths were induced (Lyte et al., 2003; Doherty et al, 2009; Gonzales et al., 2013; Sandrini et al., 2014; Yang et al., 2014; Xu et al., 2015; Boyanova, 2017; Gümüş et al., 2019) but some of them were reported to be decreased (Boyanova, 2017; Gümüş et al., 2019) in the presence of NE. In our study, both concentrations of NE decreased the growth of *P. aeruginosa* strain. However, when incubation period was prolonged, the suppressive effect was only detected at low NE concentration. It seems that the inhibitory effect of NE on the growth of *P. aeruginosa* depends on concentration and incubation period.

Host hormones have been reported to interact with several steps of pathogenesis in infectious diseases especially adhesion and invasion. Adhesion which is the first interaction between microorganisms and host tissue is very important and necessary step of the infectious process in the host. Many studies demonstrated the adhesion properties of EHEC O157:H7, adherent-invasive *E. coli, C. jejuni, S. pneumoniae, E. faecalis* strains to different host tissues (HeLa S3 cells, HT-29/B6 cells, Caco-2/TC-7 cells, A549 cells, HBMEC cells) were altered in the presence of NE (Bansal et al., 2007; Gonzales et al., 2013; Xu et al., 2015; Cambronel et al., 2020; Xi et al., 2020). It seems that in the presence of NE, whereas adhesion has been affected positively in some microorganisms (Bansal et al., 2007; Xu et al., 2015; Cambronel et al., 2020; Xi et al., 2020; Beata et al., 2021), it could be decreased in some others (Gonzales et al., 2013; Xi et al., 2020). In our study, we found out that, low concentration of NE increased the adhesion of *P. aeruginosa* strain to A549 cells. Considering this result, it is possible to suggest that hormone concentration could determine the virulence properties of microorganisms'.

It seems that, the effects of NE on the invasion to host cells were limitedly investigated. According to previous findings, invasion of *Salmonella Typhimurium*, adherent-invasive *E. coli, C. jejuni, Salmonella choleraseuis* and EHEC 0157:H7, *P. aeruginosa* strains to several cells (Peyer's patch, Caco-2 cells, HT-29/B6 cells, HCT-8 human enterocyte cells) were reported to be modulated in the presence of NE (Green et al., 2003; Brown and Price, 2008; Hegde et al., 2009; Xu et al., 2015a; Xi et al., 2020; Beata et al, 2021). According to many studies, NE acted usually as an inducer for invasion (Green et al., 2003; Brown and Price, 2008; Hegde et al., 2009; Xu et al., 2015; Beata et al., 2021). However, in our study, we did not find any effect on invasion of *P. aeruginosa* in the presence of NE.

The adhesion and invasion are defined as early stages of pathogenesis during infectious processes. In the present study, only low NE concentration was found to increase the adhesion of *P. aeruginosa* and two concentrations of NE did not modulate the invasion of bacterium.

It has been documented that catecholamines can regulate the immune system, norepinephrine, one of the catecholamines is known as a neurotransmitter that can affect the inflammation process directly or indirectly (Oberbeck, 2006; Szelényi and Vizi, 2007). Moreover, as far as is known, under stress and hard conditions, catecholamine secretions can be stimulated (Oberbeck, 2006; Osier and Dixon, 2016; Ma et al., 2020). Considering the roles of NE in both neuro-immune interactions and host-microbe communications, investigations about its influences on the infection process would make sense. According to former studies, it has been observed that the possible action mechanisms of NE are associated with up taking of iron in limited conditions, hormone-mediated induction of auto-inducers, acting as quorum sensing compounds (Lyte and Cryan, 2014; Sandrini et al., 2014; Lyte and Freestone, 2010; Freestone et al., 2007).

Conclusions

In conclusion, the purpose of our study was to investigate the influences of NE on growth, adhesive and invasive properties of a *P. aeruginosa* strain with carrying out to mimic *in vivo* conditions as possible. The results obtained from our study demonstrated that, NE has decreased the growth and increased the adhesion of *P. aeruginosa.* It should be noted that these effects were shown to depend on incubation period for growth and hormone concentration for adhesion.

Conflict of interest

There is no conflict of interest.

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CHEMICAL CONSTITUENTS OF THE ESSENTIAL OIL FROM *SALVIA VERBENACA* SSP. *CLANDESTINA* FROM ALGERIAN PRE-SAHARA

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Abstract: The essential oil obtained by hydrodistillation from the aerial parts of *Salvia verbenaca* (L.) Briq. ssp. *clandestina* (L.) Pugsl. (*Lamiaceae*) growing wild in Bou Saâda, pre-Saharan region of Algeria, was analyzed by GC-MS. Sixty-four compounds were detected, representing 95.6% of the whole oil, among them forty five compounds are identified in this sample for the first time. The essential oil of *S. verbenaca* ssp. *clandestena* showed the predominance of sesquiterpenes (56.4%) followed by monoterpene derivatives (35.5%). The main constituents were β-pinene (10.2%), spathulenol (8.7%), caryophylene oxide (6.1%), α-pinene (5.2%), germacrene D (5%) and α-gurjunene (4.9%). Chemical composition of the essential oil from our sample may be categorized as sesquiterpene and monoterpene chemotype among the four chemotypes identified for *Salvia* species.

Keywords: *Salvia verbenaca* ssp. *clandestena*, essential oil, GC-MS, β-pinene, spathulenol.

1. Introduction

Salvia, commonly known as sage, is one of the largest and most important aromatic genera of the *Lamiaceae* family, comprising about 800 species (Hao et al., 2015). It grows in Central and South America, Asia and Mediterranean regions (Tenore et al., 2010). Several traditional uses of *Salvia* species have been reported, for instance, reducing perspiration and fever, relieving digestion and spasmodic pain and treating liver disorders. Phytochemical composition of plants belonging to this genus has been previousely described, it confirmes the richness of these species in monoterpenes, diterpenoids, sterols, flavonoids and essential oils (Lu and Foo, 2002). Essential oils of plants from this genus showed antibacterial (Akin et al., 2010), antifungal (Fraternale et al., 2005), anti-inflammatory (Kamatou et al., 2005; Chan et al., 2011), anticholinesterase (Orhan et al., 2007; Kıvrak et al., 2009) and antioxidant activities (Bozin et al., 2007; Miguel et al., 2011). Furthermore, volatile oils obtained from *Salvia* species were also used in cosmetic industry, in perfumery and also as condiments (Delamare et al., 2007). Among the most known constituents identified in *Salvia* oils,

1,8-cineole, spathulenol, borneol, β-caryophyllene, germacrene D, bicyclogermacrene and caryophyllene oxide (Flamini et al., 2007; Kelen et al., 2008; Oztürk et al., 2009).

In Algeria, *Salvia* includes 23 species, five of which are endemic (Quezel and Santa, 1963). Moreover, this genus is represented in Algerian Sahara only by three species: *S. chudaei*, *S. aegyptiaca* and *S. verbenaca* (ssp. *clandestena* and ssp. *Pseudo-jaminiana*) (Ozenda, 1991). Aiming to continue our investigations on Algerian Saharan species (Smaili et al., 2011; Flamini et al., 2013, Belkassam et al., 2019, Smaili et al., 2021), the chemical characterization of the essential oil from *Salvia verbenaca* ssp. *clandestena* growing wild in Bou Saâda, pre-Saharan region in Algeria, is described.

2. Materials and methods

Plant material

Aerial parts of *Salvia verbenaca* ssp. *clandestena* (Fig. 1) were collected during the ripening stage from pre-Saharan area of Bou Saâda, South-east of Algeria. Identification of collected samples was confirmed by Pr. Tahar Smaili and a voucher sample was placed at the Department of Natural and Life Sciences, Faculty of Sciences, University of M'sila, Algeria (*S. verbenaca* ssp. *clandestena* voucher number ST/RK N°10).

Isolation of the essential oil

The air dried plant material (200 g) was coarsely cut and subjected to hydrodistillation for 2 h using a Clevenger type apparatus. Obtained essential oil was then preserved in a sealed vial at 4 °C until further analysis (Shafaie *et al*., 2019). ***GC–MS analysis***

The chromatographic analysis (GC-MS) was carried out using a Varian CP-3800 GC containing a DB-5 capillary column (30m × 0.25 mm; coating thickness 0.25 μm) and equipped with a mass spectrometry detector (Varian Saturn 2000).

Fig. 1. *Salvia verbenaca* ssp. *clandestina* plants (Original).

The analysis was performed using analytical grade helium as carrier gas at a flow rate of 1 mL/min; injector and transfer line temperature were fixed at 220°C and 240°C, respectively; the oven temperature was programmed from 60°C to 240°C at 3°C/min, using helium at 1 mL/min as carrier gas; injection of 0.2 µL of a 10% hexane solution; split ratio 1:30 (Da Silva *et al*., 2013).

Identification of volatile components

The chemical compounds of essential oil were identified based on the retention time on capillary column in comparison with injected standards; retention indices were compared to those given in the literature; computer matching identified compounds used as references (NIST 2000 and ADAMS 2007) and was also performed against homemade library of mass spectra built up from pure substances and MS literature data (Adams, 2007; Davies, 1990).

3. Results and discussions

The yield of isolated essential oil from aerial parts of *S. verbenaca* ssp. *clandestena* was 0.95% (w/w). In general, 64 compounds were identified; representing 95.6% of the whole oil, 45 compounds of them are identified in *S. clandestena* volatile oil for the first time in this study. The detailed chemical composition of the studied essential oil is summarized in **Table1.**

Table 1. Percentage composition of the essential oil from *S. verbenaca* ssp. *clandestena*.

R.t (min)	Compound name	Content %	l.r.i
3.24	(E)-2-hexenal*	0.5	854
3.45	1-hexanol*	0.2	867
4.59	α-thujene	0.3	931
4.75	**α-pinene**	**5.2**	939
5.11	camphene*	0.2	953
5.24	thuja-2,4(10)-diene	0.3	957
5.39	benzaldehyde*	0.1	961
5.72	sabinene	0.9	976
5.81	**β-pinene***	**10.2**	980
6.07	6-methyl-5-hepten-2-one*	0.3	985
6.18	myrcene	0.9	991
6.37	(E,Z)-2,4-heptadienal*	0.1	1000
6.80	(E,E)-2,4-heptadienal*	0.2	1016
6.95	α-terpinene*	0.2	1020
7.20	p-cymene*	0.3	1028
7.33	limonene	1.2	1033
7.62	(Z)-β-ocimene*	0.7	1042
7.83	benzene acetaldehyde*	0.6	1045
7.96	(E)-β-ocimene*	0.5	1052
8.33	γ-terpinene*	0.5	1063
9.39	terpinolene*	0.4	1090
9.83	linalool*	0.5	1100
10.00	nonanal*	1.1	1104
10.84	α-campholenal*	0.8	1127
11.32	trans-pinocarveol*	2.7	1143
11.58	cis-verbenol*	1.8	1145
12.29	pinocarvone*	1.8	1163

12.48	p-mentha-1,5-dien-8-ol*	0.6	1166
12.90	4-terpineol*	0.9	1179
13.47	α-terpineol*	0.3	1191
13.70	myrtenal*	3.0	1195
14.29	verbenone*	0.2	1206
14.66	trans-carveol*	0.2	1219
15.72	carvone*	0.3	1245
16.53	(E)-2-decenal*	0.4	1263
16.94	geranial*	0.2	1272
18.90	(E,E)-2,4-decadienal*	0.2	1316
20.94	(E)-2-undecenal*	0.3	1368
21.39	α-copaene	2.9	1377
21.77	β-bourbonene	1.2	1384
22.02	β-cubebene	1.0	1391
22.82	**α-gurjunene***	**4.9**	1411
23.22	β-caryophyllene	4.7	1420
23.64	β-copaene*	0.1	1433
24.38	cis-muurola-3,5-diene*	0.2	1448
24.65	α-humulene	0.8	1454
24.73	(E)-geranyl acetone*	0.4	1455
24.95	alloaromadendrene*	1.8	1461
25.07	cis-muurola-4(14),5-diene	0.8	1462
25.82	**germacrene D**	**5.0**	1482
26.11	β-selinene*	0.9	1486
26.46	bicyclogermacrene	2.2	1495
26.79	α-bulnesene*	0.4	1505
27.20	trans-γ-cadinene	4.1	1514
27.58	δ-cadinene	1.5	1525
29.33	ledol*	0.5	1565
29.73	**spathulenol**	**8.7**	1577
29.95	**caryophyllene oxide**	**6.1**	1582
30.74	Guaiol*	1.2	1595
30.98	humulene oxide II*	0.8	1608
31.23	1,10-di epi-cubenol	1.2	1615
32.23	tau-cadinol*	4.4	1642
32.77	α-cadinol*	0.7	1655
33.99	epi-α-bisabolol*	0.3	1686
	Total identified	**95.6%**	

*Compound identified for the first time in the studied essential oil. **l.r.i =** Linear retention indices (HP-5 column). **R.t =** retention time.

According to obtained results, the essential oil is mainly composed by sesquiterpene derivatives (56.4%), with hydrocarbon derivatives (32.5%) as the major sesquiterpenes fraction, largely represented by germacrene D (5%), α-gurjunene (4.9%) and β-caryophyllene (4.7%), while oxygenated sesquiterpenes (23.9%) are mainly represented by spathulenol (8.7%) and caryophyllene oxide (6.1%). The second major fraction in the studied sample is represented by monoterpenes derivatives (35.5%): Monoterpene hydrocarbons (21.8%) are principally presented by β-pinene (10.2%), α-pinene (5.2%) and limonene (1.2%) and oxygenated monoterpenes (13.7%) are mainly represented by myrtenal (3%) and *trans*-pinocarvol (2.7%).

Table 2. Chemical groups of the essential oil from *Salvia clandestena*

Chemical groups	%
Monoterpene hydrocarbons	21.8
Oxygenated monoterpenes	13.7
Total monoterpenes	**35.5**
Sesquiterpene hydrocarbons	32.5
Oxygenated sesquiterpenes	23.9
Total sesquiterpenes	**56.4**
Aldehydes	03.5
Alcohols	00.2
Total non-terpene fraction	**03.7**

Non-terpene compounds form a minor part of the sample (3.7%) and are constituted by aldehydes (3.5%) and alcohols (0.2%). The principle chemical groups found in *S. clandestena* volatile oil are summarized in **table 2.** Therefore, chemical composition of *S. clandestena* in this study may be categorized as sesquiterpene and monoterpene chemotype among the four chemotypes identified for *Salvia* species (Jassbi *et al*., 2012).

Only few data exist in literature about the chemical composition of *S. verbenaca* ssp. *clandestena* essential oil. In a study realized by Belloum *et al*. (2014), the volatile oil of the studied plant collected from a different region (Bechar) in Algerian Sahara, was found to contain germacrene D as a major constituent (20.5%), followed by α-copaene (10.4%), β-phellandrene (3.8%) and β-caryophyllene (3.8%). However, β-pinene which is the major constituent in the volatile oil of *S. verbenaca* ssp. *clandestena* in the present analysis is minor constituent of the same species growing in Bechar, permitting the hypothesis that this constituent is a chemical marker of our sample.

Similar differences were observed in the chemical composition of essential oils from *Salvia* species. In a study reported by Mohammadi *et al*. (2014) about *S. aegyptiaca* aerial parts essential oil, where the plant was collected from Bechar region in the South-west of Algeria, β-caryophyllene (10.2%), selina-4,11-diene (9.7%), bornyl acetate (8.5%) and β-gurjunene (7.6%) were identified as main constituents. Whereas the volatile oil of the same plant collected from other regions like Saudi Arabia in the study of Basaif in 2004, which showed a rich composition in 1,10-aristolene (19.3%), and also collected from Egypt in the study of El-Sawi in 2003, which confirmed the presence of trans, *trans*-farnesol, phytol, spathulenol and *cis*, *trans*-farnesol as major constituents of the oil.

Conclusions

In the present research, the chemical composition of the essential oil from aerial parts of *Salvia verbenaca* ssp. *clandestena* is reported. Obtained results were partially different from those previously described, the relatively high level of β-pinene seems characteristics of our sample and these differences may be due to the different growth habitat and environmental factors. Furthermore, the chemical composition of *Salvia verbenaca* ssp.*clandestina* volatile oil from Bou Saâda (Algeria) may be categorized as sesquiterpene and monoterpene chemotype among the four chemotypes identified for *Salvia* species. Further studies are required to evaluate biological activities of this volatile oil to know its possible applications.

Conflict of interest

None declared.

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A QUICK, SIMPLE, SENSITIVE AND SELECTIVE LC-MS/MS METHOD USED FOR THE SCREENING OF ETHEPHON, GLYPHOSATE AND AMINOMETHYLPHOSPHONIC ACID FROM WATER AND FOOD SAMPLES

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Abstract: Pesticide use has increased steadily over the years in both industrial farming and local agriculture. One of the most widely used pesticides at a global level, glyphosate, has been controversial for many years and many studies have focused on the effects of this compound on human health. Ethephon is also a controversial ripening agent used to ripen crops more quickly. A basic but crucial step in the study of the effects of their use on human health is the development of adequate analytical methodologies for the quantification of the compounds in relevant samples. For this study a quick, simple and selective method which uses LC-MS/MS was developed for the determination of ethephon, glyphosate and AMPA. The method uses selective fragment monitoring for each analyte and the internal standard, without prior analytical separation. Mobile phase used consisted of aqueous ammonium formate and methanol in isocratic elution and the sample cleanup was made using solid phase extraction (SPE). The method was validated with regards to selectivity, sensitivity, accuracy and precision in accordance with applicable guidelines. After validation the LC-MS/MS method was successfully used to determine ethephon, glyphosate and AMPA residues in ground and surface water, as well as vegetable samples.

Keywords: glyphosate, AMPA, ethephon, LC-MS, QTOF, biomonitoring, pesticide.

1. Introduction

Glyphosate (N-(phosphonomethyl)glycine) (**Fig. 1**) was first synthetized to be used as a chelating agent and chemical intermediate for other chemical compounds (US Patent Office, 1964; Nandula, 2010), and due to its chemical properties it was studied as a possible water softening agent (Nandula, 2010).

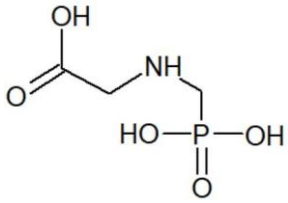

Fig. 1. Chemical structure of glyphosate

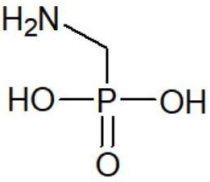

Fig. 2. Chemical structure of aminomethylphosphonic acid (AMPA)

While studying its water softening properties it was discovered that glyphoste along with other, similarly structured chemical agents, have a potential use in herbicidal products and it started being the focus in the development of a commercial herbicide instead (Nandula, 2010).

Glyphosate is an effective, broad-spectrum weedkiller and works by inhibiting an enzyme with a pivotal role in the biosynthesis of certain amino acids which play a role in the structure of plant tissue, and which are not present in animals and humans. The loss of the biological activity of glyphosate occurs when it is metabolized to aminomethylphosphonic acid (AMPA) (**Fig. 2**), which can be used as a marker of glyphosate use in biomonitoring (Schuette, 1998). In order to increase sales and profits, Monsanto also created resistant, genetically modified plants able to withstand the use of glyphosate, thus providing farmers with the opportunity to destroy weeds without affecting the crop, despite glyphosate being absorbed into the crops (Pollegioni et al., 2011).

Glyphosate is a highly water soluble, polar molecule, highly stable in solutions of various pH values and not photosensitive, making it very advantageous for farmers as a herbicide, but at the same time increasing the likeliness of its presence in crops where glyphosate is used, even after longer periods of time. Glyphosate is usually metabolized by certain types of bacteria and other microorganisms, as such its decomposition is highly dependent on the microbiota of the soil on which it is applied, the water source which it gets dissolved into or other factors (Schuette, 1998).

Although glyphosate has been shown to be present in food, groundwater and drinking water, amid a highly controversial move, the European Union extended the license for glyphosate containing products which expired in 2017 until 2022 (Myers et al, 2016; Rendón-von Osten and Dzul-Caamal, 2017; Askew, 2017). Interestingly, the limit for human exposure in the EU was increased in 2015 from 0.3 mg/kg bw/day to 0.5 mg/kg bw/day (Myers et al, 2016; Székács and Darvas, 2018), while indeed this limit is still more than three times lower compared to the USA (Myers et al, 2016).

Ethephon, 2-chloroethylphosphonic acid (**Fig. 3**), is a plant growth regulator used in to artificially accelerate ripening of fruit and vegetables.

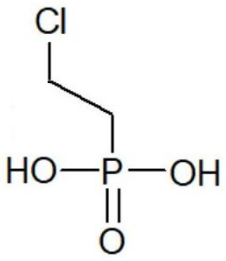

Fig. 3. Chemical structure of ethephon

It has become the most widely used ripening agent due to its positive effect on fruit coloration and ripening. It is also used in some parts of the world as an insecticide. After absorption into the plants it is metabolized into ethylene, which is a natural growth hormone for many plant species (Bhadoria, 2018).

Although ethylene is generally considered to have low toxicity in humans, the EU has currently set an acceptable limit for human exposure of 0.03 mg/kg bw/day (EFSA, 2008). In the USA however the Environmental Protection Agency (EPA) considers that there is no chronic dietary risk that might be of concern (EPA, 1995).

Due to their chemical properties and widespread use debate regarding the effects on human health of glyphosate, AMPA, ethephon and other similar substances used in agriculture have been ongoing for many years. Study results have been divisive and sometimes ambiguous, and the divisiveness has also affected the stance of regulatory agencies around the world, some regulators considering glyphosate to be safe for human use (EFSA, 2015; EPA 2020), while other organizations have labelled it as "probably carcinogenic" to humans (IARC, 2015). Due to the risk of glyphosate and its metabolite reaching the edible parts of crops and thus being consumed by the population regulatory authorities in most regions of the world limited acceptable human intake. At the same time studies on its cytotoxicity, involvement in genetic damage, tumor-cell growth and other negative health effects are being carried out (George, 2010; Suárez-Larios, 2017). There have been studies showing that the use of ethephon can also have negative health effects on humans, ranging from loss of appetite, diarrhea, stomach aches, but in some cases can also result in dermatotoxicity and hepatotoxicity (Bhadoria, 2018; EFSA, 2008).

Among the controversies it has become growingly important to have adequate methodologies to be able to measure human exposure to these compounds. The development of such methods poses a number of challenges however with regards to analytical performance and sample preparation depending on the sample matrices studied (soil, ground water, food samples, drinking water, blood/plasma, urine, tissues etc.).

The aim of the present study is to propose a fast, simple and selective LC-MS/MS method for the determination of ethephon, glyphosate and AMPA in ground and surface water, as well as vegetable samples, for quick screening purposes.

2. Materials and methods

Reagents and solutions

Glyphosate, AMPA and ethephon were acquired from LGC (Augsburg, Germany), glyphosate-2-^{13}C,^{15}N was acquired from MilliporeSigma (Burlington, USA). HPLC grade methanol was acquired from Honeywell (Seetze, Germany), ammonium formate was acquired from VWR International (Radnor,

USA). Ultrapure water was sourced from a Millipore Direct-Q 3 (Milford, USA).

Equipment

An LC-MS system composed of a Perkin Elmer (Waltham, USA) FX-10 UHPLC coupled with an AB Sciex (Framingham, USA) quadrupole-time-of-flight (QTOF) 4600 mass spectrometer was used. Other equipments: Eppendorf (Hamburg, Germany) 5430R centrifuge; Radwag (Radom, Poland) XA 523Y analytical scale; Velp Scientifica (Usmate Velate, Italy) vortex mixer; JP Selecta (Barcelona, Spain) Ultrasons H-D ultrasonic bath; Eppendorf Research Plus (Hamburg, Germany) automatic pipettes. Solid phase extraction was performed using Macherey-Nagel Chromabond HLB 1ml/100mg (30µm) cartridges (Düren, Germany).

Sample collection

A total number of 8 food samples (tomatoes and bell peppers) and 10 water samples were collected during a two week period in April-May 2021 in order to be analyzed for residual glyphosate, AMPA and ethephon content.

A number of four samples of tomatoes (*Solanum lycopersicum*) of different varieties were acquired: two were bought from supermarkets, having originated from Spain and Italy respectively according to the labels; one was bought from a local farmer's market in Targu Mures (Romania), being labeled as locally grown; one was collected in a local vegetable garden in Bistrita-Nasaud county (Romania). Out of the four pepper (*Capsicum annuum*) samples, two were bought from supermarkets, with origins being labeled as Italy and Romania; one was bought from a local farmer's market in Targu Mures, being labeled as locally grown; one was collected in a local vegetable garden in Bistrita-Nasaud county. All samples were stored at 5 ^{o}C until analyzed.

For the water sample analysis, a total ten samples were collected, with one being local Targu Mures tap water as reference, another being water from a local creek in Targu Mures collected near the evacuation point of a nearby farmer's market. The other eight samples were collected near a local farm and vegetable garden in Bistrita-Nasaud county, with four collection points: well upstream from the vegetable garden; creek collecting rainwater immediately adjacent to the vegetable garden and bordering it; well downstream from the vegetable garden; collecting creek at the limits of the property approximately 500 m distance from the vegetable garden. For each of these four collection points a sample was taken within 48 hours of using glyphosate-based products and after one week from using the herbicide. After collection, all samples were stored at -20 ^{o}C until analyzed.

LC-MS analysis

Stock solutions of glyphosate, AMPA and ethephon were prepared with concentrations of 10 µg/ml with aqueous 20 mM ammonium formate as solvent. These stock solutions were used to prepare a mixed standard solution with a concentration of 1µg/ml of each analyte, also in aqueous 20 mM ammonium formate, further used to prepare the working calibration solutions in the same solvent with concentrations ranging between 0.5-100 ng/ml of each analyte, with a total of 8 calibration levels. Using the mixed standard solution quality control working solutions of three different concentration levels were prepared for validation purposes with concentrations of 1.5 ng/ml (QCA), 40 ng/ml (QCB) and 80 ng/ml (QCC), respectively. A solution of isotopically labeled glyphosate-2-^{13}C,^{15}N was prepared to be used as internal standard (ISTD) for all solutions, with a concentration of 6.5 µg/ml

using aqueous 20 mM ammonium formate as solvent.

Calibration standards for the calibration curves were prepared by mixing 990 µl of the appropriate working solution with 10 µl of internal standard solution and performing solid phase extraction similarly to the sample solutions. Blank ISTD solutions containing only internal standard were also prepared by mixing 990 µl of mobile phase with 10 µl of internal standard solution. The concentration of internal standard in the final solutions was 65 ng/ml.

For the water samples cleanup was performed by mixing 990 µl of sample with 10 µl of internal standard solution and performing solid phase extraction using HLB cartridges. The cartridges were conditioned with methanol followed by a conditioning step with a mixture of 1:1 methanol:1% aqueous formic acid (v/v), after which the sample was loaded onto the cartridge and the eluent collected and filtered through 0.45 µm polypropylene syringe filters. Tomato and bell pepper food samples were each mashed up, and 100 mg of the mashed and homogenized samples were extracted in 990 µl of aqueous 20 mM ammonium formate mixed with 10 µl of internal standard through ultrasonication for 15 minutes, centrifuged at 10000 rpm for 3 minute and the supernatant extracted using the same SPE method as for water samples.

Detection of analytes was carried out in multiple reaction monitoring (MRM) mode for all analytes and the internal standard, after negative electrospray ionization. The ionization parameters were used for the ionization source were: spray voltage: -4500V, vaporizer temperature: 550 ^{o}C, ion gas source 1: 30, ion gas source 2: 30, curtain gas: 10, declustering potential: -10. The following fragment ions were monitored for each compound: fragments m/z 150.05, 123.04, 79.05 and 63.05 from parent m/z 167.98 at a collision energy (CE) of -14 V for glyphosate; 79.05 and 63.05 from parent m/z 109.95 at a collision energy (CE) of -20 V for AMPA; fragments m/z 107.02, 99.05 and 79.04 from parent m/z 142.90 at a collision energy (CE) of -9 V for ethephon; and fragment ions m/z 152.05, 125.05, 81.05 and 65.05 from m/z 169.95 at CE -14 V for the detection of the internal standard. An injection volume of 10 µL of each solution was injected into the LC-MS system with a total sample run-time of 3 minutes per solution. No chromatographic separation was used and the mobile phase was composed of methanol and aqueous 20 mM ammonium formate solution with a ratio of 40:60 (v/v) in isocratic conditions, at a constant flow rate of 0.1 mL/min.

3. Results and discussions

Method validation

The method was validated with regards to sensitivity, selectivity, linearity, accuracy, precision, carryover and post preparative stability in accordance with current regulatory guidelines (EMA, 2012). A bioanalytical method can be considered selective and sensitive enough for the application it was designed for if any interfering peaks in blank samples have peak areas lower than 20% of the peak areas at the lower limit of quantification (LLOQ) for the analytes and lower than 5% of of the peak area of the internal standard. To prove the selectivity of the method blank samples containing only mobile phase were analyzed. For ethephon, although initially the LLOQ was set at the same concentration as for glyphosate and AMPA (0.5 ng/ml), due to issues obtaining acceptable selectivity, the first calibration standard was eliminated from each analytical run and the final LLOQ for ethephon was set at 1ng/ml.

Table 1. Selectivity and sensitivity for glyphosate, AMPA, ethephon and internal standard

Analyte	**Mean Blank Area**	**LLOQ Area**	**Selectivity (%)**
Glyphosate	17.287	460.856	3.75
AMPA	22.222	266.448	8.34
Ethephon	0.000	10955.702	0.00
Glyphosate-2-^{13}C,^{15}N	338.675	12539.783	2.70

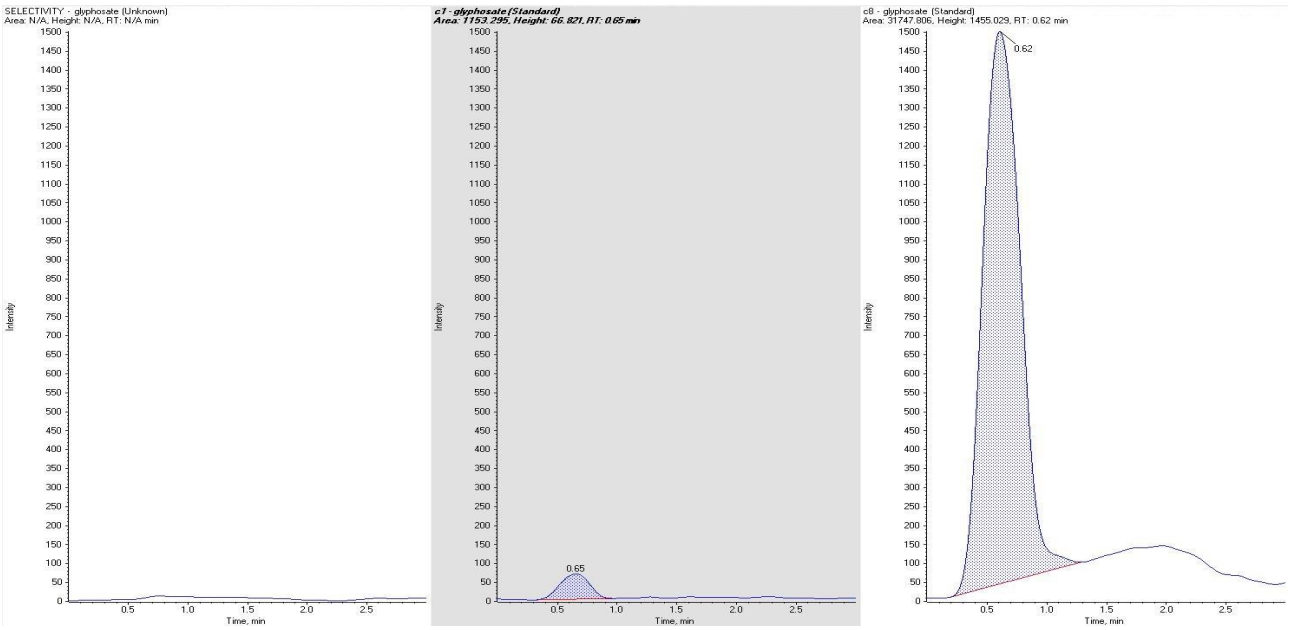

Fig. 4. Extracted chromatograms for glyphosate - blank solution, LLOQ, ULOQ solution

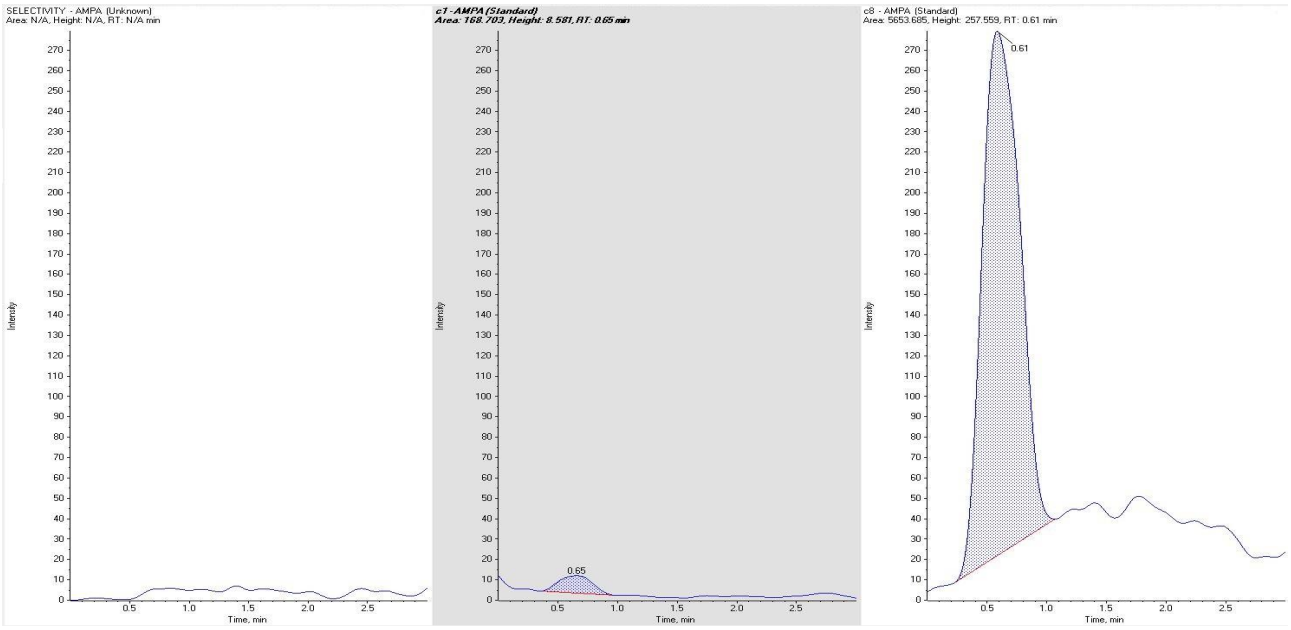

Fig. 5. Extracted chromatograms for AMPA - blank solution, LLOQ, ULOQ solution

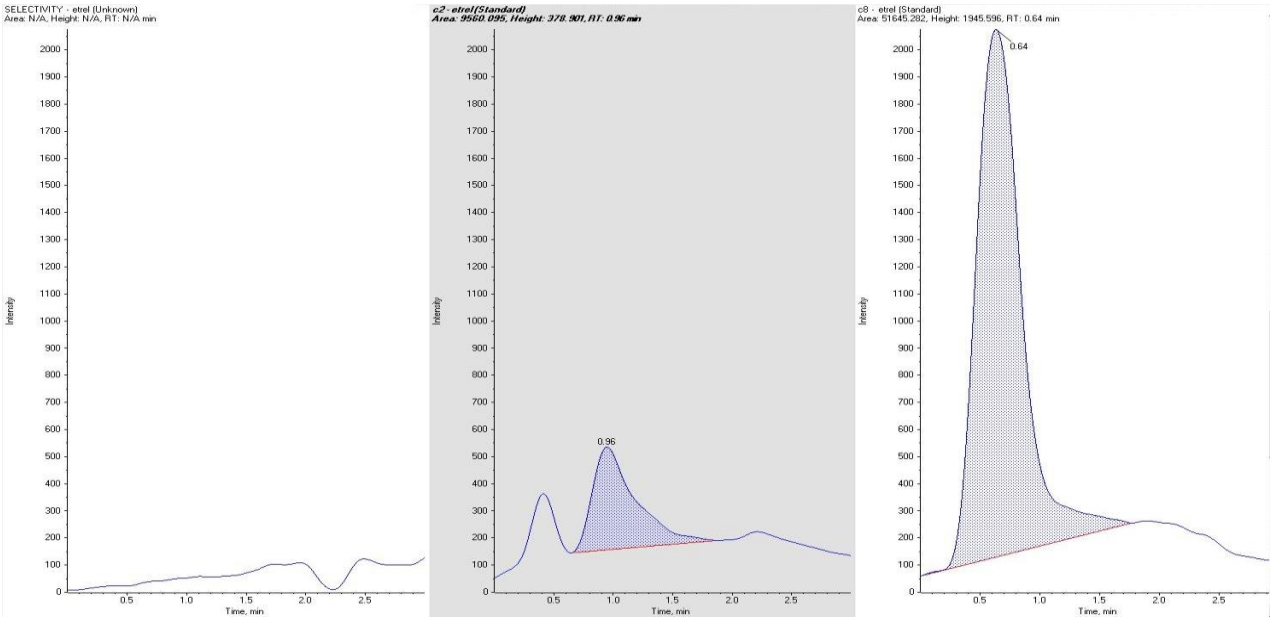

Fig. 6. Extracted chromatograms for ethephon - blank solution, LLOQ, ULOQ solution

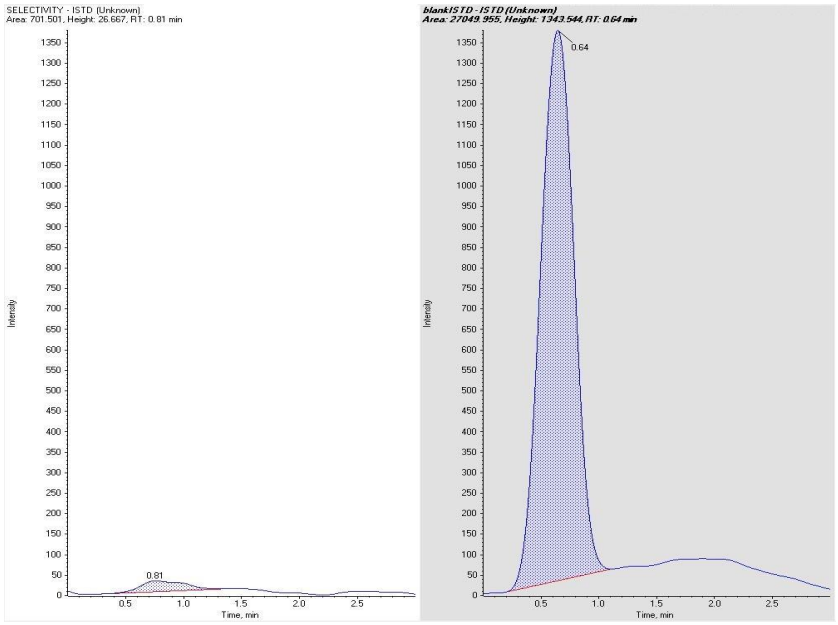

Fig. 7. Extracted chromatograms for internal standard - blank solution, blankISTD solution

No interfering peaks with area greater than 20% of analyte peak areas at the limit of quantification (LLOQ) were detected (**Fig. 4-7**) for any of the analytes. Results for selectivity testing are presented in **table 1**. A freshly prepared calibration curve was injected into the LC-MS system for each analytical run, both during validation and sample analysis, in order to assess linearity of the method. For each run of the analytical method during method validation the calibration curves were linear for all analytes, with no more than 2 of the 8 calibration standards needing to be excluded due to low accuracy residuals (**Fig. 8**). The coefficient of correlation (R) was higher than 0.99 for all calibration curves during both validation and sample analysis, and the values for coefficients of correlation and residuals are presented in **tables 2-4**. Accuracy and precision of the method were verified at three different

concentration levels (QCA, QCB and QCC), both within a single analytical run and between different analytical runs, by analyzing five different quality control samples at each concentration level. The results for accuracy, calculated as percentage bias from the theoretical value, and precision, calculated as coefficient of variation, were within acceptance limits of ±15% and are presented in **tables 5-7**. The carryover of the analytical method when injecting a blank sample after a high concentration standard solution (ULOQ) was below the threshold provided by validation guidelines, with interfering peaks areas in the blank solutions injected for carryover testing being lower than 20% of the area of analytes at LLOQ and lower than 5% of the area of the internal standard. Thus it was concluded that no significant carryover is present when using the analytical method. Results for carryover testing are presented in **table 8.**

Table 2. Coefficient of correlation for validation calibration curves and residuals - glyphosate

	Accuracy %				
Nominal concentration ng/ml	**Analytical run 1**	**Analytical run 2**	**Analytical run 3**	**Analytical run 4**	**Analytical run 5**
0.500	-17.2	8.3	-19.2	-12.2	4.3
1.000	9.2	-9.4	*70.4**	0.5	*-77.6**
5.000	7.4	5.9	5.1	13.6	-10.4
10.000	0.2	*-25.2**	6.5	7.3	12.5
25.000	-4.7	3.0	14.5	0.1	-0.7
50.000	2.5	7.4	11.3	3.7	3.4
75.000	-2.3	-12.0	-8.1	-5.1	5.0
100.000	0.8	4.6	-11.5	-7.8	-6.3
Coefficient of correlation					
R	0.9991	0.9964	0.9940	0.9974	0.9972

** excluded due to inaccuracy >15% (20% for LLOQ)*

Table 3. Coefficient of correlation for validation calibration curves and residuals - AMPA

	Accuracy %				
Nominal concentration ng/ml	**Analytical run 1**	**Analytical run 2**	**Analytical run 3**	**Analytical run 4**	**Analytical run 5**
0.500	-11.3	-3.0	-9.7	4.0	9.9
1.000	*97.5**	*-56.4**	*115.5**	-6.2	-11.6
5.000	13.5	4.4	6.2	-7.8	-3.0
10.000	14.4	-3.8	10.8	13.5	12.1
25.000	12.5	3.9	11.6	8.6	11.2
50.000	9.2	8.6	6.6	10.4	12.5
75.000	-11.3	*-33.6**	-9.8	-12.6	-3.6
100.000	-13.8	-8.1	-10.2	-1.4	-13.8
Coefficient of correlation					
R	0.0992	0.9977	0.9950	0.9946	0.9936

** excluded due to inaccuracy >15% (20% for LLOQ)*

Table 4. Coefficient of correlation for validation calibration curves and residuals - ethephon

	Accuracy %				
Nominal concentration ng/ml	**Analytical run 1**	**Analytical run 2**	**Analytical run 3**	**Analytical run 4**	**Analytical run 5**
0.500	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*
1.000	5.8	-7.0	-5.6	-18.3	7.1
5.000	*-98.2**	5.0	-10.0	-8.9	-4.8
10.000	4.9	*-15.6**	*-36.8**	1.1	3.4
25.000	-3.0	-2.7	12.1	2.7	*23.0**
50.000	-11.4	8.7	-3.3	7.0	-5.3
75.000	4.2	-12.7	-2.4	-3.6	8.5
100.000	9.8	10.6	0.9	-1.3	-2.6
Coefficient of correlation					
R	0.9957	0.9929	0.9985	0.9989	0.9981

** excluded due to inaccuracy >15% (20% for LLOQ)*

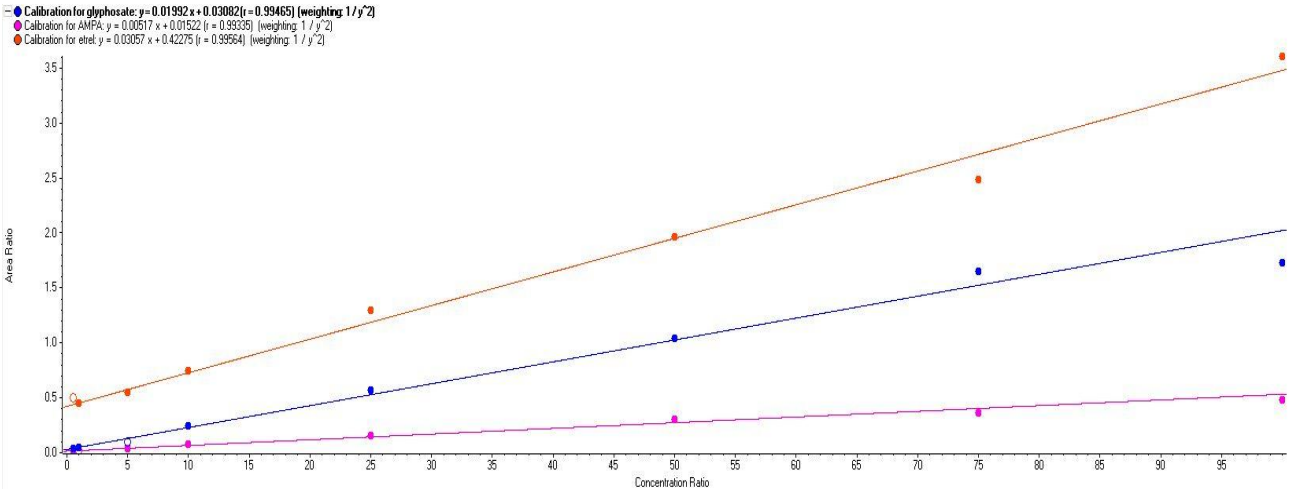

Fig. 8. Calibration curves for glyphosate (blue), AMPA (magenta) and ethephon (orange)

Table 5. Accuracy and precision of the analytical method - glyphosate

Within run accuracy and precision				**Between run accuracy and precision**			
Nominal conc. ng/ml	**Mean measured conc. ng/ml**	**Precision %**	**Accuracy %**	**Nominal conc. ng/ml**	**Mean measured conc. ng/ml**	**Precision %**	**Accuracy %**
1.500	1.460	12.5	-2.6	1.500	1.628	6.9	8.5
40.000	40.380	1.6	0.9	40.000	42.023	9.6	5.1
80.000	79.342	1.3	-0.8	80.000	84.522	7.3	5.7

The stability of analyte containing solutions, tested and proven by reinjecting quality control samples prepared on a previous day after being kept in the autosampler at room temperature, allows for preparation of standard calibration solutions and samples at any time and injecting them into the LC-MS system within 24 hours after being prepared. The results of the autosampler stability testing were within the ±15% acceptance limits and are presented in **table 9**.

Table 6. Accuracy and precision of the analytical method - AMPA

Within run accuracy and precision				Between run accuracy and precision			
Nominal conc. ng/ml	**Mean measured conc. ng/ml**	**Precision %**	**Accuracy %**	**Nominal conc. ng/ml**	**Mean measured conc. ng/ml**	**Precision %**	**Accuracy %**
1.500	1.698	4.1	13.2	1.500	1.644	5.5	9.6
40.000	44.890	4.9	12.2	40.000	43.356	11.7	8.4
80.000	73.936	4.8	-7.6	80.000	77.046	4.0	-3.7

Table 7. Accuracy and precision of the analytical method - ethephon

Within run accuracy and precision				Between run accuracy and precision			
Nominal conc. ng/ml	**Mean measured conc. ng/ml**	**Precision %**	**Accuracy %**	**Nominal conc. ng/ml**	**Mean measured conc. ng/ml**	**Precision %**	**Accuracy %**
1.500	1.549	8.5	3.2	1.500	1.658	4.2	10.5
40.000	43.692	13.7	9.2	40.000	36.428	8.7	-8.9
80.000	86.530	7.1	8.2	80.000	89.561	2.0	12.0

Table 8. Carryover for glyphosate, AMPA, ethephon and internal standard

Analyte	**Mean Blank Area**	**Mean LLOQ Area**	**Carryover (%)**
Glyphosate	0	639.907	0.00
AMPA	8.042	173.054	4.65
Ethephon	0	8535.563	0.00
Glyphosate-2-^{13}C,^{15}N	184.653	16216.661	1.14

Table 9. Autosampler stability for glyphosate, AMPA and ethephon

Analyte	**QC sample conc. ng/ml**	**Mean calculated conc. ng/ml**	**Accuracy (%)**
Glyphosate	1.5 (QCA)	1.56	3.7
	40 (QCB)	44.46	11.1
	80 (QCC)	86.52	8.2
AMPA	1.5 (QCA)	1.57	4.7
	40 (QCB)	45.47	13.7
	80 (QCC)	82.05	2.6
Ethephon	1.5 (QCA)	1.42	-5.1
	40 (QCB)	42.74	6.8
	80 (QCC)	83.46	4.3

Sample analysis

All food and water samples collected were analyzed for glyphosate, AMPA and ethephon content. Results of the analysis showed that some of the samples analyzed had quantifiable amounts of glyphosate and AMPA residue, but at the same time ethephon was not identified in any of the samples. The values measured in each of the samples for the studied analytes are presented in **tables 10-11**.

As a proof-of-concept study, the current research fulfills its purpose as the LC-MS method developed can successfully be used to analyze water and food samples for residues after glyphosate use. Glyphosate and its metabolite, AMPA, were detected, although at low concentrations, in some commercially acquired food samples, but also in food samples cultivated with previous use of glyphosate-based herbicides. As expected, in the immediate vicinity of crops where glyphosate is sprayed to herbicide residues of the compound and its metabolite can be found in ground and surface waters, even a few days after use, due to the slow decomposition in the environment. Ethephon was not detected in any of the samples, which in the case of food samples might be either due to the substance not being used, or due to the quick metabolization once absorbed into plants. In the case of the collected water samples, as no ethephon is known to have been used in the area of collection, it was not expected to detect the substance, however the research offers a method which can be used to detect ethephon in ground or surface waters, in cases when the substance is used, as stability in these types of samples should be higher and detection possible after a longer period of time.

Table 10. Results for food samples analyzed for glyphosate, AMPA and ethephon residues

Sample	Glyphosate ng/mg	AMPA ng/mg	Ethephon ng/mg
Food sample 1	-	-	-
Food sample 2	56.4	54.7	-
Food sample 3	11.0	-	-
Food sample 4	148.1	130.1	-
Food sample 5	31.5	37.3	-
Food sample 6	15.4	10.8	-
Food sample 7	-	-	-
Food sample 8	-	-	-

Table 11. Results for water samples analyzed for glyphosate, AMPA and ethephon residues

Sample	Glyphosate ng/ml	AMPA ng/ml	Ethephon ng/ml
Water sample 1	-	-	-
Water sample 2	5.69	13.63	-
Water sample 3	21.77	26.89	-
Water sample 4	11.89	8.92	-
Water sample 5	-	-	-
Water sample 6	1.44	-	-
Water sample 7	-	-	-
Water sample 8	17.38	18.48	-
Water sample 9	1.08	-	-
Water sample 10	-	-	-

As finding truly blank matrices can be difficult in certain situations, due to the widespread and rarely reported use of glyphosate and ethephon, it was not possible to test for matrix effect during the study, however the matrix effect can be determined on a case-by-case basis using the matrix of a sample and spiking it with standard solution, analyzing the spiked sample together with the unspiked matrix and standard solution. This enables a more accurate quantitation of the analytes, in case it is deemed necessary, and sufficient amounts of the appropriate matrix are available.

While many analytical methodologies described in literature use derivatization in order to achieve quantification (Trass, 2014), similar LC-MS methods for the underivatized determination of glyphosate and its metabolite, as well as ethephon, have been described in literature, in some cases being showcased by equipment and reagent vendors and manufacturers (Shimelis, 2019; Schreiber and Jin, 2016; Ulrich and Ferguson, 2021). Compared to existing methods, the lack of analytical separation for the method developed during this study, although also having some drawbacks, has the advantage of a very short runtime of only 3 minutes per sample analyzed. This enables the quick screening of a large number of samples in a very short timeframe, while using a quick and simple solid phase extraction which is used by most other methods also. The method developed thus has similar performances, in some cases even higher sensitivity, compared to other similar existing methods, while enabling a slightly faster analysis.

Conclusions

The method developed can be used for screening purposes in the analysis of food and water samples for glyphosate and ethephon use. Although the method uses no chromatographic separation, an aspect which could be improved in order to avoid interferences in certain cases, the highly sensitive and selective QTOF mass spectrometric detection offers the performance needed for such an application.

While after use the detection of ethephon might be more difficult due to the rather quick metabolization to ethylene after absorption into plants, it could still be useful to perform screening in water samples and even food samples, since the method enables the detection of very low residual concentrations. At the same time, the use of glyphosate in certain crops leaves behind a detectable trace of either glyphosate or its main metabolite, or both compounds, which can ultimately easily be detected and quantified.

Conflict of interest

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

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