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Chemical analysis of *Viloa odorata* L. (Fam. Violaceae) and the efficacy of its essential oil against some stored product insects

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Abstract

The current experiment was carried out to assess the insecticidal activity of sweet violet (*Viola odorata*) essential oil against three major stored product insects (*Tribolium castaneum, Rhyzopertha dominica* and *Sitophilus oryzae*). The chemical composition of sweet violet essential oil in different cuts (first, second and third cut) of *V. odorata* grown in Al-Gharbia governorate, Egypt was determined. For the first cut, 73.825 % linolenic acid was the main component, while for second cut, 61.000 % linolenic acid was the main component and for the third cut, the main component was 75.419 % linolenic acid. The biomass yield was changed on different cuts. In the first cut, the yield was 15.500 ton/acre while it was 10.300 and 6.800 ton/acre for the second and the third cut respectively. In mixing with medium experiment, *Triboleum castaneum* was the most tolerant insect against violet absolute essential oil while after one day of exposure *Rhizobirtha dominica* was the most sensitive insect with LC_{50} of 53730 mg/kg. After 24 h of exposure in thin film experiment, *R. dominica* was found to be the most sensitive insect with LC_{50} of 475 mg/L. At the highest concentration (15000 mg/kg) there were no emerged adults for *T. castaneum* and *S. oryzae* while there were a mean of 0.33 emerged adults for *R. dominica*. The reduction was 100 % at the highest concentration for *S. oryzae* and *T. castaneum* while it was 99.53 % for *R. dominica*.

Keywords

Rhyzopertha dominica; sweet violet; *Tribolium castaneum*; *Sitophilus oryzae*; stored products

Introduction

Aromatic plants are very important and economic in the agricultural production, therefore, this study is focused on sweet violet plants. Previous studies investigated the use of essential oils in aroma therapy or in medicine. Stored grains are the most important product from crops worldwide especially wheat. Wheat grain represents the main source of protein in poor countries (1). Wheat crop suffers from many stress factors such as drought (2, 3), salinity (4, 5), heat (6), plant pathogens (7, 8) and insects (9). Storage of wheat and other stored products are very important. Countries which import wheat need protecting the crop during the whole year especially during the summer months with high temperatures and humid weather.

The high levels of temperature and relative humidity are the best conditions for stored-product pests (10, 11). *Tribolium castaneum*, *Sitophilus oryzae* and *Rhyzopertha dominica* are the most important and major pests. The infestation of these insects can cause complete weight loss of stored wheat grain within 6 months of storage (12-14). Controlling these pests needs a huge quantity of synthetic insecticides and fumigants. Regarding the heavy use of synthetic insecticides and fumigants, world is already facing many problems such as pollution of the environment, increasing of the costs of crop production, pest resistance and harmful impacts to non-target organisms especially natural enemies as well as toxicity to users due to direct contact (15).

Essential oils are important products which are produced from aromatic plants during secondary metabolism and give the plant its characteristic odor and flavor (16). These oils have become the topic of various experiments and researches aiming to assess their insecticidal activities. Essential oils have little effect on the environment or on human health (17). The effectiveness of plant essential oils and other plant-based products may help in controlling pests on many plants and this can help to discover different pesticides from plants (18-20). Fast degradation of natural products in the environment and low toxicity to mammals and non-target organisms make plant-based natural products ideal pesticides for the control of insect pests (21, 22). Many aromatic plant species are cultivated as economic crops in Egypt (13). In Egypt, the sweet violet plant blooms in winter and the farmers there, plant sweet violet mainly for its leaves while flowers are a secondary product. *V. odorata* has been used traditionally as a cure to respiratory and inflammatory conditions. Violet is an economic plant especially in Al-Gharbia governorate, Egypt. Violet plants can resist and tolerate insect infections during their mature stage, which might be related to their essential oils (10, 11). Thus, this work was designed to study the chemical composition of violet essential oil during three cuts and the contact toxicity. The study also aimed to study the effect of the essential oils of violet on the progeny of *T. castaneum, S. oryzae* and *R. dominica*.

Materials and Methods

Wheat grains and flour were used for rearing adults of *Rhyzopertha dominica*, *Sitophilus oryza* and *Tribolium castaneum*. Wheat grains and flour were heated at 50 ºC for 6 h to get rid of any prior insect infestation. Six 500 mL glass jars were used, wheat grains were provided in 4 jars (250 g/jar) and flour were provided in 2 jars (250 g/jar). 100 adults of *R. dominica*, *S. oryzae* and *T. castaneum* were transferred to the jars. All cultures were kept at 28 ± 2 ºC and 65 ± 5 % R.H, with light: dark photoperiod of 16:8 h. The newly emerging adults (0-7 days) were collected by sieving the diets. Adult insects, used for all bioassays were of mixed sexes.

Violet herb, *Viola odorata* L. (Fam. Violaceae), grown in Al-Gharbia governorate, Egypt. (30º 59' 23.6" N 30º 53' 18.6" E) was harvested for 3 cuts, the first, second and third cuts were after 180, 250 and 320 days from plantation. The extraction of crude violet essential oils was carried out at the Hashem Brothers Company according to the following protocol (13). The violet herb was dried in the air for 24 h. One-ton of dried violet herb was loaded in a still (5000 L^3 capacity) and the extraction was carried out using hexane. 3000 L of hexane were added till the herb was covered completely for 2 h. The solvent was then concentrated till the volume reduced to 50 L. The previous process was repeated 2 times with different soaking times 2 and 12 h, concentrated hexane (150 L) was left for 12 h to be cooled and then filtered using filter paper. The filtered solvent was concentrated under temperature of 50 °C and vacuum -1 bar in order to remove all the solvent and obtain the concentrate. The concentrate was dissolved in 99 % ethyl alcohol after cooling and filtered for 5 times to completely remove the wax. All the solvent (ethyl alcohol which contains absolute) was concentrated at 70 ºC and vacuum of 1 bar as alcohol will be removed to have only absolute (13, 21).

From our experience and regarding our experiments in the field with Hashem Brothers Company for Essential oils and Aromatic Products, violet does not succeed in all soil structures and its chemical analysis and biomass yield changes according to the soil structure. The soil structure was studied as the following in the Table 1.

Table 1. Soil analysis of the experimental site.

EC	SAR	Cations				Anios				AN	AP	AK
		Na	Ca	Mg	K	CO_3	HCO_3	Cl	SO4			
3.55	9.95	23.85	7.44	4.25	0.42	0.01	5.52	16.72	13.55	65.50	11.56	257.00
4.22	10.90	28.65	8.85	5.05	0.43	0.03	5.01	20.01	17.90	67.08	12.05	264.00
1.65	6.75	10.95	3.43	1.93	0.25	0.05	5.57	7.68	3.35	70.30	10.53	267.00

Violet absolute oil

To obtain violet absolute oil, the violet herb was air dried for 24 h, then One-ton of dried violet herb was loaded in a still (5000 L capacity) and the extraction was done using hexane (3000 L) according to (13, 23).

GC-MS analysis

The constituents of essential oils were analyzed by GC/MS and the compounds were identified with the help of other studies (24, 25) in the Analytical Laboratory of Hashem Brothers Company for Essential Oils and Aromatic Products. The constituents of violet absolute analyzed by gas chromatography-mass spectrometry (GC/MS) (HP5890, USA) system with an HP column (60 m x 0.25 mm, 0.25 µm film thickness) (HP-5ms). The initial and maximum temperature was 60 °C and 250 °C respectively for 65.3 min. The injector temperature was 240 °C. Relative percentage amounts were calculated from peaks total area. The compounds were identified by matching the mass spectra data with those held in a computer library (Wiley 275.L) (24, 25).

Bioassay

Thin film technique

Contact toxicity bioassay was carried out by exposing tested insects to a thin film layer of oil in a petri dish (9 cm) (26). The Petri dishes were treated by 1 mL of diluted essential oil in acetone as a solvent for each dish at five concentrations (250, 500, 1000, 2000 and 4000 mg/L) and left to dry. After complete dryness, 10 adults of each tested insect were placed in each treated Petri dish. The same number of insects also was confined on petri dish treated only with acetone and served as control. Each treatment and control repeated 3 times. Mortality was recorded after 24 h of exposure and corrected by Abbott's formula (27). The LC_{50} values for all insecticides were calculated by the method (28).

Mixing with feeding medium

The 5 concentrations (25000, 50000, 100000, 200000 and 400000 mg/kg) of violet oil were diluted in acetone and added to 20 g of uninfected wheat grains for *R. dominica* and *S. oryzae* and 20 g of flour for *T. castaneum* in 170 mL glass jars. Jars were mechanically shaken to ensure complete mixing with wheat grain. A glass Jar contains 20 g of wheat grain treated with acetone served as a control. Concentrations and control were replicated 3 times (14). The treated grains were allowed to dry at room temperature and 10 unsexed adults of the tested insects were transferred to each jar and covered with screw cap. Mortality counts were recorded after 24, 48, 72, 96 h, a week and 2 weeks after exposure. Data were adjusted according to another study (27). The slope, LC_{50}, LC_{90} and confidence limit values were recorded according to Finney's analysis (29).

Effect on progeny

A laboratory study was designed to assess the effect of violet oil on the progeny of *R. dominica, S. oryzae* and *T. castaneum.* Batches of (50 g) of uninfected wheat grains for *R. dominica* and *S. oryzae* and 50 g of flour for *T. castaneum* were placed in 170 mL jars and treated with 4 concentrations (2500, 5000, 10000 and 15000 mg/kg), then were infested with 20 adults of tested insects for each jar. After 2 weeks, all insects were removed (14). Jars were kept in an incubator at 26 ± 1 °C and 65 ± 5 % R.H. The untreated grains and flour were used as control, the treatments were repeated 3 times. The newly appeared adults were noted for 2 weeks and the adult reduction (%) was recorded as the following:

$$\text{Reduction (\%)} = \frac{MNEC - MNET}{MNEC} \times 100$$

MNEC : mean No. of emerged adults in the control,

MNET: mean No. of emerged adults in the treatment

Data analysis

The mortality percentage was analyzed using a one-way ANOVA and LSD test at P = 5 %, SPSS software program version 23 was used. The (LC_{50}) 50 % lethal concentrations, slope and 95 % confidence limits (CL) were calculated based on the method (29) and the significant difference between LC_{50} values was assessed based on 95 % CL overlapping.

Results

Violet absolute chemical analysis

In the current study, first, second and third cuts of violet herb were extracted and the essential oil was analyzed and only violet absolute essential oil was tested on insects.

Table 2. Chemical analysis of the first cut of *Violo odorota* absolute essential oil.

Serial No.	R.T. (min)	component	%
1	2.9	Heptane	0.140
2	2.94	Pentane	0.200
3	3.041	Hexane	0.755
4	3.107	Cyclopenatnol	0.350
5	3.244	Octane	0.050
6	3.723	Benzenemethanol	0.095
7	7.003	Linalool	0.450
8	8.096	Trans-2-*cis*-6-nonadienal	0.280
9	9.042	Alpha terpineol	0.100
10	19.659	Acetic acid	0.130
11	20.114	2-Cyclohexen-1-one	0.470
12	20.853	decane	0.325
13	21.886	Tetradecanoic acid	0.165
14	23.332	Octacosyl trifluoroacetate	0.110
15	23.895	Pentadecanoic acid	0.185
16	25.082	Hexadecanoic acid	0.100
17	25.508	*cis*-9-hexadecanoic acid	0.225
18	26.283	Hexadecanoic acid	16.455
19	26.385	Palmitic acid	0.444
20	28.219	Methyl linolenate	0.135
21	28.342	Methyl ester	0.490
22	29.556	Linolenic acid	73.825
23	35.808	alpha Linolenate	0.225
24	37.813	Docosane	0.230
25	39.633	Squalene	0.555
26	40.565	Docosane	0.410
27	43.846	Eicosane	0.486
28	44.739	Vitamine E	0.510
29	52.738	Chola-5,22-dien-3-ol	0.745
30	53.364	Sitostenone	1.555
Biomass yield/acre			15.500 ton

Data in Table 2 cleared that a total of 30 components were identified with accounting of 99.79 %. Linolenic acid was the main component with 73.825 % followed by hexadecanoic acid with 16.455 % and sitostenone with 1.555 %.

Data in Table 3 presented that a total of 43 components were identified with accounting of 99.985 %. Linolenic acid was the main component with 61.00 % followed by Alpha linolenate with 16.030 % and Palmitic acid with 15.600 %.

Data in Table 4 cleared that a total of 41 components were identified with accounting of 99.99 %. Linolenic acid was the main component with 75.419 % followed by

Table 3. Chemical analysis of the second cut of *Violo odorota* absolute essential oil.

Serial No.	R.T. (min)	Component	%
1	2.903	Propanoic acid	0.040
2	2.941	Pentane	0.015
3	3.043	Hexane	0.380
4	3.11	Cyclopentanol	0.135
5	3.247	3-hexen-1-ol	0.050
6	3.377	Octane	0.040
7	3.516	Linalool	0.065
8	3.577	Benzenethanol	0.110
9	3.726	Trans-2-cis-6-nonadienal	0.060
10	7.006	2-nonenal	0.420
11	7.447	Acetic acid	0.141
12	8.101	Alpha terpineol	0.500
13	8.231	Acetic acid	0.131
14	8.348	2-cyclohexen-1-one	0.090
15	9.043	spiro (4.5)decane	0.070
16	19.665	Tetradecanoic acid	0.139
17	20.129	Undecanoic acid	0.455
18	20.852	Pentadecanoic acid	0.300
19	21.9	Hexadecanoic acid	0.132
20	23.332	cis--9-hexadecanoic acid	0.050
21	23.903	palmitic acid	0.135
22	25.079	9-octadecenoic acid	0.095
23	25.531	heptadecanoic acid	0.017
24	26.417	9,12-octadecanoic acid	15.600
25	27.406	methyl linolenate	0.104
26	27.759	Phytol	0.080
27	28.226	alpha linolenate	0.125
28	28.354	linolenic acid	0.520
29	28.718	eicosaptaenoic acid	0.141
30	29.574	Docosane	16.030
31	30.167	methyl ester	61.00
32	32.427	linolenic acid	0.080
33	34.897	Docosane	0.080
34	35.697	tricontyl actate	0.140
35	35.762	Squalene	0.320
36	37.823	Ethyl 6,9,12-hexadectrienoate	0.220
37	39.152	Docosane	0.001
38	39.639	Vitamin E	0.239
39	40.573	decane-10-one	0.224
40	43.857	Sitostenone	0.401
Biomass yield/acre		10.300 ton	0.210

Table 4. Chemical analysis of the third cut of *Violo odorota* absolute essential oil.

Serial No.	R.T. (min)	component	%
1	2.898	pentanol	0.180
2	2.938	pentane	0.164
3	3.038	Hexane	0.530
4	3.104	Cyclopentanol	0.360
5	3.24	Butane	0.045
6	3.37	Butanol	0.064
7	3.509	Butene	0.110
8	3.555	Hexen-ol	0.085
9	4.742	Thiourea	0.090
10	5.263	Hexen-1-ol	0.120
11	5.404	3-Hexenol	0.186
12	6.991	linalool	0.610
13	8.086	Trans-2-cis-6-nonadienal	0.380
14	8.216	1-Menthone	0.358
15	8.445	cyclohexnone	0.326
16	9.029	Alpha terpineol	0.085
17	9.792	beta citronellol	0.925
18	10.404	Geraniol	0.587
19	10.83	2,6-octadiene	0.186
20	11.469	geranoil formate	0.138
21	19.051	1H-cuclopropa(a)naphthalene	0.515
22	20.106	2-cyclohexene-1-one	0.220
23	20.582	geranyl tiglate	0.150
24	25.528	Palmitoleic acid	0.433
25	26.256	hexadecanoic acid	11.720
26	26.385	Oleic acid	0.019
27	28.339	methyl linoleate	0.234
28	29.825	linolenic acid	75.419
30	35.823	Ethyl linolenate	0.586
31	37.817	Eicosane	0.185
32	38.033	Isophthalic acid	0.298
33	38.529	cyclohexaneethanol	0.151
34	39.631	squalene	0.145
35	40.047	9,12,15-octadecatrienal	0.145
36	40.444	citronellyl isobutyrate	0.481
37	40.592	1,5,9-Decatrienene	1.560
38	40.956	3,7-dimethyl acetate	0.234
39	41.108	ethanol	0.629
40	43.845	Eicosane	0.385
41	53.255	stigmast-4-en-3-one	0.400
Biomass Yield			6.800 ton

hexa decanoic acid with 11.720 % and 1,5,9-decatrienene acid with 1.560 %.

Bioassay

Mixing with medium

Data in Table 5 showed that, *T. castaneum* was the most tolerant insect against violet absolute essential oil. After 1 and 2 days of exposure violet absolute had no effect against *T. castaneum* while after one day of exposure *R. dominica* was the most sensitive tested insect with LC_{50} of 53730 mg/kg.

Table 5. Mixing with medium effect of *Viola odorata* essential oil against adults of *Rhizobirtha dominica, Sitophilus oryzae* and *Tribolium castaneum*.

Exposure period (day)	LC_{50} (mg/Kg)	95 % Confidence Limits	Slope value	Chi²	LC_{90} (mg/kg)
Rhizobirtha dominica					
1	53730	36641 – 72131	0.98	4.50	1066936
2	29832	17633 – 40140	1.18	0.02	358611
3	19846	8082 – 30019	1.05	0.88	326509
4	11478	3283 – 19679	1.13	0.83	156069
7	--	-- --	--	--	--
14	--	-- --	--	--	--
Sitophilus oryzae					
1	685333	334151 – 5575933	1.48	3.88	5024701
2	238078	190462 - 297597	1.69	13.68	1361496
3	86336	69068 - 107920	3.00	47.49	230420
4	45914	36731 – 57392	3.02	10.25	121644
7	20274	16219 – 25342	2.64	17.20	61799
14	--	-- --	--	--	--
Triboleum castaneum					
1	--	-- --	--	--	--
2	--	-- --	--	--	--
3	258960	198475 – 470653	3.16	3.05	658664
4	227364	166165 – 401099	1.62	0.37	398632
7	66020	58405 – 72994	3.89	0.82	72994
14	38304	30643 – 47880	2.56	4.80	121110

Thin film toxicity

The presented results in Table 6 showed that after 24 h of exposure, *R. dominica* was the most sensitive tested insect with LC_{50} of 475 mg/L followed by *S. oryzae* with LC_{50} of 1973 mg/L, while *T. castaneum* was the most tolerant tested insect with LC_{50} of 2087 mg/L.

Table 6. Thin film effect of *Viola odorata* essential oil against adults of *Rhizobirtha dominica, Sitophilus oryzae* and *Tribolium castaneum* after 24 h of exposure.

Insect	LC_{50} (mg/L)	95 % Confidence limits	Slope value	Chi²	LC_{90} (mg/kg)
Rhizobirtha dominica	475	223 – 680	2.04	3.65	1632
Sitophilus oryzae	1973	1654 – 2307	2.08	1.96	8128
Triboleum castaneum	2087	1670 – 2609	3.25	7.44	9671

Effect on progeny

Data in Table 7 showed that all concentrations of violet absolute essential oil had a reduction effect against the three tested insects. At the highest concentration of 15000 mg/kg, there were no emerged adults for *T. castaneum* and *S. oryzae* while there were 0.33 emerged adults for *R. dominica* was obtained. The reduction was 100 % at the highest concentration for *S. oryzae* and *T. castaneum* while it was 99.53 % for *R. dominica*.

Discussion

In the current study, results indicated that violet absolute essential oil has insecticidal activity against the three tested stored product insects (*T. castaneum*, *S. oryzae* and *R. dominica*). Our results indicated that the main component is linolenic acid and also contains linalool which are affected on the respiratory system of stored product insects and also effect on acetylecolenesterase (9-11, 13). The contact toxicity refers to the essential oil properties which effect on the insect's cuticle and also effects on the respiration pores. Essential oils have a significant effect on stored grain insect's progeny and that regarding its repellent activity and the volatile properties which effect on the insect antenna and the chemical receptors (10, 11). The contact

Table 7. Means of F1-progeny adults emerged from *Rhizobirtha dominica, Sitophilus oryzae* and *Tribolium castaneum* exposed to *Viola odorata* essential oil at different concentrations compared to control treatment.

Insect	Oils concentrations (mg/kg)	Mean no. of adults emerged ± SE	% Reduction[a] in F1-progeny
Rhizobirtha dominica	Control	70.00 ± 2.3[a]	0
	2500	3.00 ± 1.52[b]	95.71
	5000	2.66 ± 1.20[b]	96.20
	10000	0.66 ± 0.33[b]	99.05
	15000	0.33 ± 0.33[b]	99.53
Sitophilus oryzae	Control	42.0 ± 3.46[a]	0
	2500	1.66 ± 0.33[b]	96.05
	5000	0.66 ± 0.33[b]	98.43
	10000	0.00 ± 0.00[b]	100
	15000	0.00 ± 0.00[b]	100
Tribolium castaneum	Control	16.33 ± 2.30[a]	0
	2500	1.33 ± 0.66[b]	91.85
	5000	0.33 ± 0.33[b]	97.98
	10000	0.00 ± 0.00[b]	100
	15000	0.00 ± 0.00[b]	100

Means followed by the same letter(s) in each column are not significantly different (P=0.05; LSD test).

a % Reduction in F1-progeny production = [Cn- Tn] / [Cn] x 100, where, Cn is the number of newly emerged insects in the untreated (control) jar and Tn the number of insects in the treated jar

toxicity of *Viola odorata* essential oil against stored product pests was studied against *Cryptolestes ferrugineus* (Stephens) (30). Their results of the chemical composition of *V. odorata* grown in Kashmir found that, the main component was butyl-2-ethylhexylphthalate with (30.10 %). The chemical composition of *V. odorata* was studied (31) and found that, the main component is ɑ̣-linalool (15.81 %). The *V. odorata* extract toxicity was evaluated on the *Agonoscena pistaciae* (Hemiptera: Psyllidea) and recorded the mortality rate (32). Their results showed that the *V. odorata* extract was effective on *A. pistaciae* as pesticide and recorded the mortality percentage. The toxicity effect of *V. odorata* was recorded against *T. castaneum* and produces wide kinds of cyclotides, including kB1 (kalata B1) and cyO2 (cycloviolacin O_2) (33, 34). *V. odorata* can protect itself against mite attack in mature stage and in summer while in winter it has a heavy attack of mites on its leaves. In summer and before mature stage violet has many attacks of worms specially army worm. One acre of violet produces average 25 tons of biomass which can produce about 16.5 kg of violet absolute essential oil in 3 cuts. Regarding to its chemical composition, *V. odorata* oil can be used as antimicrobial and anticancer (34). Many studies proved that linolenic acid is safe to mammals and can be used in medicine and aroma therapy. Regarding their study, *V. odorata* oil is safe to mammals and human health, as it can be used safely in stored grain and flour protection. Also in agreement with the current study, essential oils and its complex compositions are already used in aromatherapy and for centuries aromatic plants was used as medicinal plant as traditional products in medicine system (12, 13). Many people use aromatic formulas as treatments for many illnesses such as those that affect the central nervous system (35). Most of these essential oils components have low molecular weights. The effect of essential oils on stored product insect had mortality effect on *Sitophilus oryzae* and *R. dominica* and also affected their progeny. The chemical structure of violet oil was studied (10, 11) and the result was in agreement with current study. The current study can be used as a reference to the companies and the farmers who working on violet (*Viola odorata*). Result cleared the chemical composition of violet absolute and showed that chemical composition of violet essential oil differs in each cut and that may be due to the weather conditions. Biomass decreasing throws the cuts (15.500, 10.300 and 6.800 ton/acre) for the first, second and third cut respectively.

Conclusion

In the current study, violet absolute can be used as stored grain protectants in IPM program. Regarding to the chemical analysis, linolenic acid was the main component with 73.825 %, 61.00 % and 75.419 % for the first, second and third cut respectively. *Rhizobirtha dominica* was the most sensitive insect in all toxicity experiments. Violet absolute essential oil with the concentration of 1500 mg/kg achieved 100 % reduction for *Tribolium castaneum* and *Sitophilus oryzae* while the same concentration achieved 99.53 % for *R. dominica*. New researches should be carried out to decrease the cost of using essential oil in plant protection. Current study can be used as a reference and a guide to farmers and companies work in violet plantation and producing essential oils.

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Authors' contributions

DME and AMA performed the experiments and wrote the manuscript and participated in manuscript revising and editing. AMA and WMA conducted experimental methodology, participated in data analysis and representation and participated in manuscript revising and editing. AMA, KhA and YH conceived and designed research, provided the used chemicals, provided practical guidance and participated in manuscript revising and editing. AMA, FMAK, DME and KhA provided the chemicals, provided practical guidance and participated in manuscript revising and editing revising and editing. AMA, FMAK, DME, WME, YH and KhA conceived and designed the research, conducted experimental methodology, participated in data analysis and representation. AMA, FMAK, DME and WME suggested the research point, investigated the article, conceived and designed the research. All authors read and approved the article.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

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