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RESEARCH ARTICLE

IN VITRO ANTI-LEISHMANIAL ACTIVITY AGAINST CUTANEOUS *LEISHMANIA* PARASITES AND PRELIMINARY PHYTOCHEMICAL ANALYSIS OF FOUR YEMENI MEDICINAL PLANTS

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Abstract

Objective: *Cutaneous leishmaniasis* is one form of leishmaniasis that chiefly infected the poor sections of the society. The prototypical therapeutic interventions in vogue are handicapped due to toxicity and an alarming increase in drug resistance. So, the aim of our study was to assess the anti-leishmanial activity of *Euphorbia cactus* Ehrenb, *Euphorbia ammak* Forssk, *Euphorbia inarticulate* Schweinf, and *Pergularia tomentosa* L.
Methods: The extracts of plants were prepared by maceration method and by Soxhlet extractor. The extracts were dried and re-dissolved in 2% dimethyl sulfoxide (DMSO) 1% solvent. *Leishmania spp*. cells were then tested with serial concentrations (15.6 $\mu g ml^{-1}$ to 500 $\mu g\ ml^{-1}$) of the extracts, using the 3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. All experiments were performed in triplicate and analyzed by ANOVA test. The optical density values as measured by Enzyme-Linked Immunosorbent Assay (ELISA) were used to calculate the IC_{50} values.
Results: The results indicated that the methanolic latex extract of *Euphorbia cactus* Ehrenb, *Euphorbia ammak* Forssk had potent anti-leishmanial activity against the promastigotes of *Leishmania* spp. based on a dose-dependent response analysis. The IC_{50} values for *Euphorbia cactus* Ehrenb and *Euphorbia ammak* Forssk after 24 h incubation against *Leishmania* spp. promastigotes were less than <15.6 $\mu g\ ml^{-1}$. Furthermore, the phytochemical analysis of methanolic extracts showed the presence of alkaloids, phytosterols, phenols, saponins, and flavonoids.
Conclusion: In conclusion, the present study reveals that latex extract of *Euphorbia cactus* Ehrenb and *Euphorbia ammak* Forssk contain active compounds that have anti-leishmanial activity, which could serve as an alternative agent in the treatment of *Cutaneous leishmaniasis*, but further studies would, therefore, be needed to assess the activity of these materials of this plants *in vivo* clinical response and study their toxicity on cell lines.
Keywords: Anti-leishmanial activity, *Euphorbia cactus* Ehrenb, *Euphorbia ammak* Forssk, *Euphorbia inarticulate* Schweinf, *Pergularia tomentosa* L, Yemeni medicinal plants.

INTRODUCTION

Cutaneous leishmaniasis (CL), one clinical form of leishmaniasis, a term referred to skin clinical symptoms caused by several species of obligate intra-monocular phagocytic cells protozoan parasites produce a skin ulcer that heals spontaneously in most cases leaving an unsightly scar . These parasites belong to the genus *Leishmania*, that transmitted by the bite of a female phlebotomine sand fly[1-4]. *C. leishmaniasis* is still one of the world's most neglected disease that significant morbidity worldwide and shows a worrying increasing trend. CL is endemic in large areas of the tropics, subtropics, and the Mediterranean basin[5]. In Yemen**,** this disease is endemic and the most prevalent skin infectious diseases[6,7]. To date progression in developing an effective vaccine against CL has not been successes and chemotherapy is the only effective

way to treat the disease. However, current therapy is toxic, expensive, have severe side effects, as well as it emerges a resistance to drugs. Therefore, there is a great and urgent need for developing a new and safe anti-leishmanial drug[8-11].

Investigation bioactive compounds from plants that used medicinally are regarded as one of the strategies to discover new drugs for leishmaniasis[12]. The World Health Organization (WHO) has estimated that approximately 80% of individuals rely on traditional medicines for their primary health care needs[13,14]. In different cultures and countries, many plants are used in the form of powders, crude extracts or infusion to treat several diseases including parasitic diseases without any scientific evidence of efficacy. In Yemen, there is a rich tradition of the use of herbal medicine for the treatment of various diseases, including inflammations, infections and other diseases[15-18].

Despite the worldwide spread of *C. leishmaniasis* and the significant morbidity that caused by this disease in the world as well as in Yemen, its current drugs have limitations. So the investigation of plants that are used in folk medicine may have prognostic value to discover new and safe cutaneous leishmanicidal drug. The present study conducted to offer a scientific basis for the traditional use of *Euphorbia cactus* Ehrenb, *E. ammak* Forssk, *E. inarticulate* Schweinf, stems and leaves of *Pergularia tomentosa L* as cutaneous leishmanicidal, this may be regarded as future promising phytotherapeutics in the treatment *C. leishmaniasis.* The objectives of this study were to evaluate the *in vitro* cutaneous anti-leishmanial activity of *E. cactus Ehrenb, E. ammak* Forssk, *E. inarticulate* Schweinf, stems and leaves of *P. tomentosa L* extracts. Also carrying out preliminary phytochemical screening of those plant extracts.

Table 1: List of the selected plants that used in the investigation.

Plant species (family)	Part used [a]	Site of collection
E. cactus Ehrenb (Euphorbiaceae)	Lat.	North Taiz
E. ammak Forssk (Euphorbiaceae)	Lat.	North Taiz
E. inarticulate Schweinf (Euphorbiaceae)	Pha.	North Taiz
P. tomentosa L (Asclepiadeceae)	S,L	North Taiz

a- S. :Stem, Pha. : phylloclades, L:Leaves , Lat.: Latex.

SUBJECTS AND METHODS

Plant materials

Four selected plants (*E. cactus* Ehrenb, *E. ammak* Forssk, *E. inarticulate* Schweinf, stem and leaf of *P. tomentosa* L (Table 1) commonly used in Yemen by traditional healers for the treatment of *C. leishmaniasis* and other skin diseases were collected from Taiz governorate of Yemen in September 2016, and botanical identification was by Dr. Hassan Ibrahim botanist at the Botany section, Biology department, Faculty of Science, Sana'a university, Sana'a (Yemen).

Plant preparation

Stems and leaves of *P. tomentosa* L and phylloclade of *E. inarticulate* Schweinf were collected, washed, sliced, weighed and sundried under the shade at room temperature. After complete drying, they grinded to a coarse powder in electrical blender. The dried crude plants were maintained in dark vials and stored at -20°C until used[19,20]. The latex of *E. cactus* Ehrenb and *E. ammak* Forssk were collected in clean glass bottles and were kept in the refrigerator (4–8°C) until extraction[20].

Table 2: Percentage extraction yield of plant extracts.

Plant name	Crude methanolic extract (g)	Extracted amount (g) extraction yield (%)
E. cactus Ehrenb	5.24	206 (2.54%)
E. ammak Forssk	11.215	309 (3.629%)
E. inarticulate Schweinf	32.870	350 (9.39%)
P. tomentosa L leaves	29	300 (9%)
P. tomentosa L Stems	43.98	300 (14.66%)

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Plants extraction

The dried plants were extracted successively with MeOH (80%) by using a Soxhlet extractor**.** The obtained extracts were filtered through a Whatman-1 filter paper. The filtrates were dried by evaporation on a rotary evaporator below 45°C and freeze dryer to give the crude dried extract. All extracts were stored at -20°C until used, the yield obtained are shown in Table 2. Latex was extracted by maceration using MeOH (80%). The latex was soaked (1 ml) in 10 ml of solvent in a stoppered container with frequent agitation at room temperature. After maceration the soaked latex were filtrated through a Whatman-1 filter paper. The filtration was dried by a rotary evaporation and freeze drier to give the crude dried extract. The crude dried extract of latex were stored at –20°C, the yield obtained are shown in Table 2.

Anti-promastigote assay

Patient selection and *Leishmania* spp. isolation

Following clinical diagnosis by dermatologist, and confirmed by laboratory. *Leishmania* spp. were isolated from the patient with *C. leishmaniasis* infection. Skin lesions of the patients were cleansed with 70% ethanol before sample scraping. These preparations were stained with Giemsa and examined under a light microscope with magnification (×1000), and it was inoculated in Nicolle-Novy-McNeal (NNN) culture medium. The culture tubes were kept in an incubator at 25°C for 5-10 days. *Leishmania* spp. Promastigote that observed during microscopy transferred to RPMI-1640 and incubated at 25°C for mass cultivation medium. Patient who included in this study were: (1) patient with infection not exceed 6 months, (2) patient did not used drugs or herbs, and (3) Heavy infection.

Determination of 50% promastigote growth inhibitory concentration (IC_{50}) of the plant extracts

Stock solution of crude extracts was prepared in 2 % (DMSO)/deionized water at 10 mg/10 ml. RPMI-1640 medium were distributed in each well of a 96 well

plate. The extract solutions (100 µl) were serially diluted down each lane of 96-well plate with medium. Then, the growing cells at 10^6 promastigotes/ml were added to each well to give final six concentration of plant extracts 15.6 µg ml, 31.25 µg ml^{-1}, 62.56 µg ml^{-1}, 125 µg ml^{-1}, 250 µg ml^{-1}, 500 µg ml^{-1} (two folds dilutions).

Table 3: *In vitro* activity of *E. cactus* Ehrenb methanolic extracts on *Leishmania* spp. after 24h.

Concentration (µg/ml)	Parasite inhibition (mean±SD)*	Inhibition %
15.6	0.034±0.045[a,b]	80.5
31.25	0.034±0.046[a,b]	80.5
62.56	0.034±0.037[a,b]	84.32
125	0.034±0.018[a,b]	92.37
250	0.034±0.0176[a,b]	92.54
500	0.034±0.017[a,b]	92.79
Untreated control	0.034±0.0236	

Mean ±SD is OD values of 3 wells. * Average ± standard division,
[a]No significant difference compared to each other ($p>0.05$).
[b]Significant difference compared to untreated wells ($p<0.05$)

The final concentration of DMSO was not be higher than 1% (v/v) as this concentration did not affect the parasite growth rate, mobility, morphology[21] in all experiments. A Pentostam (Albert David Limited-India) was used as positive control at 10 µg ml^{-1}, untreated media were used as negative control, and DMSO alone was used as solvent control. 96-well micro-plates were incubated at 26ºC. After the incubation, the effect of the isolated extracts on cell viability was assessed using the 3-(4,5-dimethylthaz-olk-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which measures the metabolic activity of mitochondria[22]. In which, 20 µl of MTT solution (5 $mgmL^{-1}$) (SIGMA) was added to each well and incubated for 4 h at 26ºC. The test principle "Tetrazolium salts are cleaved to formazan dye by cellular enzyme mitochondrial succinate dehydro-genase (only in the viable promastigites)". Finally, the MTT solubilization solution was added to each well to dissolve the insoluble purple formazan product into coloration solution and incubated for another 10 min. The absorbance was measured at 570 nm for each well, using an ELISA reader[23,24]. The live promastigotes percentage inhibition ratio were calculated as described by Bansal *et al.*,[25].

Inhibition Rate (I%)

Data were presented as mean±SD of triplicate experiments for each well. Mediam inhibitory concentration value (IC_{50} value), the concentration that decreased parasite growth by 50% was calculated using linear regression analysis (dose–response analysis) associated with 95% confidence interval. Lower IC50 value indicates greater anti-leishmanial activity.

***Leishmania* spp. counting**

20 µl of cells from the culture were taken and placed in 1.5 ml Eppendorf tube containing 20 µl of Eosin (1%) then mixed. After that 10 µl of cells were placed on haemocytometer (Thoma slide). Total 5 squares were counted then the result numbers were multiply by 100000 so we can get the number of cells in 1 ml of culture.

Table 4: *In vitro* activity of *E. ammak* Forssk methanolic extracts against *Leishmania* spp. after 24h.

Concentration (µg/ml)	Parasite inhibition (mean±SD*)	Inhibition %
15.6	0.088±0.006[b]	62.71
31.25	0.089±0.004[b]	62.28
62.5	0.084±0.002[b]	64.4
125	0.075±0.001[b,c]	68.22
250	0.067±0.007[b,c]	71.61
5006	0.068±0.001[b,c]	71.18
Untreated control	0.008±0.236	

Mean ±SD is OD values of 3 wells. *Average ± standard division,
[b]significant difference compared to untreated wells ($p<0.05$).
[c]significant difference compared to 15.6, 31.25, and 62.5 µg/ml

Preliminary qualitative phytochemical screening

The dried extracts were tested to identify alkaloids, carbohydrates, glycosides, fixed oil and fats, anthraquinones, phenolic compounds and tannins, phytosterols, proteins, saponins, gum and mucilage. A preliminary phytochemical analysis was carried out using tube-test reaction and thin-layer chromatography (TLC) as described by Banu and Cathrine[19].

Statistical Analysis

Data are presented as mean±SD of triplicate experiments, and the plant extract concentration required for 50% inhibition *in vitro* (IC_{50}) calculated by dose-response analysis associated with 95% confidence interval by Graph pad prism 7 Demo.

Ethical Consideration

Ethical clearance for the study was taken from the Faculty of Medicine and Health Sciences Research Review Committee. Informed consent was taken from the patients before the collecting specimens.

Table 5*: In vitro* activity of *E. inerticulata* Shweinf extracts against *Leishmania* spp. for 24h.

Concentration (µg/ml)	Parasite inhibition (mean±SD*)	% Inhibition
15.6	0.164±0.033[b]	30.5
31.25	0.155±0.016[b]	34.32
62.56	0.154±0.015[b]	34.74
125	0.142±0.012[b]	39.83
250	0.141±0.01[b]	40.25
500	0.119±0.008[b,c]	49.59

Mean ±SD is OD values of 3 wells. * Average ± standard division,
[b]significant difference compared to untreated wells ($p<0.05$).
[c]significant difference compared to 15.6 µg/ml ($p<0.05$).

RESULTS

The percentages of extraction yield for each investigated plant were summarized in Table 2. The results of anti-Promastigote assay (*in vitro* test) for the five plant extracts against promastigotes stages of *Leishmania* spp. are summarized in Table 3 to Table 7. Data in Table 8 elucidate that the highest antileishmanial activity was obtained from *E. cactus* Ehrenb and *E. ammak* Forssk with IC_{50} value < 15.6

µg ml^{-1}. The leaves and stems of *P. tomentosa* L and *E. innerticulata* Schweinf extracts showed inactive antileishmanial activity (IC_{50} value > 500 µg ml^{-1}).

Table 6*: In vitro* activity of *P. tomentosa L.* leaves methanolic extracts against Leishmania spp. for 24h.

Concentration (µg/ml)	Parasite inhibition (mean±SD*)	% Inhibition
15.6	0.172±0.03[a,b]	27.11
31.25	0.168±0.022[a,b]	28.81
62.56	0.155±0.021[a,b]	34.32
125	0.151±0.001[a,b]	36.01
250	0.146±0.021[a,b]	38.13
500	0.146±0.033[a,b]	38.13
Untreated control	0.008±0.236	

Mean ±SD is OD values of 3 wells. * Average ± standard division,
[a]No significant difference compared to each other (p>0.05).
[b]Significant difference compared to untreated wells (p<0.05)

Phytochemical analysis and thin layer chromatography results-

Phytochemical analysis of extracts indicated the presence of various bioactive components. Compounds, such as alkaloids were found in the five plant extracts. However, fixed oils, fats and Anthra-quinones were absent in all extracts, Phytosterols were found in the four plant extract and in *E. ammak* Forssk extract was absent. Glycosides, Gum and Mucilage were found only in *E. cactus* Ehrenb and *E. ammak* Forssk, proteins were found only in *E. cactus* Ehrenb and *E. inarticulate* Schweinf.

Table 7: *In vitro* activity of *P. tomentosa L. stems* methanolic extracts against Leishmania spp. for 24h.

Concentration (µg/ml)	Parasite inhibition (mean±SD*)	Inhibition %
15.6	0.001±0.207	12.28
31.25	0.001±0.203	13.98
62.5	0.008±0.189[b,c]	19.91
125	0.004±0.191[b,c]	19.06
250	0.004±0.171[b,c]	27.54
5006	0.012±0.151[b,c]	36.01
Untreated control	0.008±0.236	

Mean ±SD is OD values of 3 wells. * Average ± standard division,
[b]Significant difference compared to untreated wells (p<0.05).
[c]significant difference compared to 15.6 and 31.25 µg/ml (p<0.05).

However, Saponines were found only in *E. cactus* Ehernb, *E. ammak* Forssk, stem and leaf of *P. tomentosa L*, and carbohydrates were found in *E. inerticulata* schweinf, stem and leaf of *Pregularia tomentosa* L (Table 9). The TLC analysis showed spots of determined constitutes in each extract (Table 10).

DISCUSSION

The first choice drug for treatment of *C. leshmaniasis* is Pentostam which has toxic side effects. Also, no vaccine is available to cure this disease. Most of the studies directed towards plants that is used traditionally as potential source of new alternative medicines. Some of the drugs obtained from plants used in the treatment of diseases caused by protozoan include alkaloids quinine obtained from the plant genus *Cinchora* and artemisinin obtained from the plant genus *Artemisia annua* and both of them used in the treatment of malaria. As well as, emetin obtained from the plant genus *Cephaelis* used in treatment of ameobiasis[26]. Findings of current study showed that the methanol latex extract of *Euophobia cactus* Ehrenb (Euophrbiaceae) had choice antileshmanial activity (IC_{50}<15.6 µg ml^{-1} against promastigotes). There was no previous studies reported about its biological activities against *Leishmania* spp. to compare with, so current results appear to be one of the first that studied its activity. The methanol latex extract of *E. ammak Forssk* showed a good antileishmanial activity (IC_{50}<15.6 µg ml^{-1} against promastigotes). Current obtained data is compatible with data reported by Abdel-Sattar *et al.*, against *Leishmania infantum* which found to inhibit the growth of *Leishmania infantum* with IC_{50} value<24.05 µg/ml[27]. These findings confirmed that latex of *Euophobia cactus* Ehrenb and *E. ammak* Forssk could be considered as having promising antileishmanial activity.

Table 8: IC_{50} values (µg/ml) for promastigotes growth inhibition of the methanol extracts of five plants.

Plant extracts	µg/ml
E. cactus Ehrenb	<15.6
E. ammak	<15.6
E. innerticulata Schweinf	>500
P. tomentosa L. L	>500
P. tomentosa L. S	>500

IC_{50}=Inhibitory concentration 50%, L= leaves, S= stems.

The methanol extract of *E. inarticulate* Schweinf phylloclades showed no antileishmanial activity (IC_{50}>500 µg ml^{-1}). Although, there was no previous studies reported about its biological activities against *Leishmania* spp. to compare with, so current results appear to be one of the first that study its antileishmanial activity. The leaves and stems extracts of *P. tomentosa L* and *E. inarticulate* Schweinf showed no antileshmanial activity (IC_{50}>500 µg/ml), while several studies reported good activity of these plants against fungi[28,29]. Flavnoids may be the active compound in *E. ammak* Forssk against *Leishmania* spp. in current study. Current findings can be confirmed by the finding of Das *et al.*,[30] in which they tested the antileishmanial activity of flavonoids against topo-isomerase I of *Leishmania donovani*. They illustrated that these compounds inhibited topoisomerase I which subsequently inhibit the relegation step in parasite growth[30]. Phenolic, saponins, and alkaloids that we detected in current study may be they are the active compounds responsible for antileishmanial activity in *E. ammak* Forssk and *E. cactus* Ehrenb. Kayser *et al.*,[31] in their comprehensive review of antiparasitic drug development view phenolic, saponins, and alkaloids have inhibition activity against *Leishmania* parasite[31,32]. Phytochemical results of leaves and stems of *P. tomentosa* L indicated the presence of saponins, phytosterols, phenolic compounds, tannins, carbohydrates, alkaloids, and flavonoids, except

coumarins only present in stems. The phytochemical results of leaves and stems of *P. tomentosa* L in current study is in agreement with Hassan *et al*., and Shinkafi in Nigeria, except glycosides, and anthraquainins were not present in extracts of *P. tomentosa L*[28,29]. The absent of glycosides, and anthraquainins in plant *P. tomentosa L* might be due to the environmental factors, such as altitude, temperature, and locations as described by Zidorn and Stuppner in which they referred these differences in components is due to the environmental factors[33].

Table 9: Qualitative phytochemical analysis of plant extracts by chemical method.

Constituents	Test	E.C	E.A	E.I	P.L	P.S
Alkaloids	Mayer's test	+	+	+	+	+
	Wanger's test	+	+	+	+	+
Carbohydrates	Bendict's test	-	-	+	+	+
Fixed oil and fats	Spot test	-	-	-	-	-
Glycosides	Salkowski's test	+	+	-	-	-
Anthraquinones	Borntrager's test	-	-	-	-	-
Phenolic compounds	Ferric chloride test	-	-	+	+	+
and Tanins	Mg and HCL reduction test	-	-	+	+	+
Phytosterols	Libermann-Burchard's test	+	-	+	+	+
Proteins	Biuret test	+	-	+	-	-
Saponins	Foam test	+	+	-	+	-
Gum and Mucilage	Gum and Mucilage test	+	+	-	-	-

+= signify presence , -= signify Absence, E.C= *E. cactus*, E.I= *E. inerticulata*, E.A= *E. ammak*, P.L= *P. tomentoa L* leaves, P.S= *P. tomentoa L* stems

Table 10: TLC investigation of methanol extract of five plant extracts.

Constituent	Solvent System	Plant extract	No. spots	Rf values
Anthraglycoside	EtoAc: MeOH: water (100:13.5:10)	E.C	--	--
		E.A	--	--
		E.I	1	0.62
		PL	1	0.58
		PS	3	0.2
				0.42
				0.56
Bitter principles	EtoAc: MeOH: water (100:13.5:10)	E.C	2	0.05
				0.69
		E.A	2	0.072
				0.73
		E.I	2	0.53
				0.8
		PL	--	--
		PS	3	0.29
				0.56
				0.87
Flavonoid	EtOAc: formica.: glacial acetica.: water (100:11:11:26)	E.C	--	--
		E.A	1	0.68
		E.I	2	0.6
				0.72
		PL	1	0.52
		PS	1	0.52
Saponins	$CHCL_3$:glacial acetic a.: MeOH: water (64:32:12:8)	E.C	1	0.14
		E.A	1	0.14
		E.I	1	0.5
		PL	2	0.12
				0.46
		PS	2	0.3
				0.4
Coumarins	Diethyl ether: toluene (1:1)	E.C	1	0.59
		E.A	1	0.70
		E.I	--	---
		PL	--	---
		PS	3	0.14
				0.63
				0.73

TLC: thin layer chromatography, EtoAc: ethyle acetate, MeOH: methanol, $CHCl_3$: chloroform, a: acid, RF: retention factor,---:absent, *EC: E. cactus Ehrenb, EA: E. ammak, EI=E. inerticulata shwenf , PL:P. tomentosa L. leaves, PS: P. tomentosa L. Stem* TLC: (a) anth (anthraquinones) after sprayed with 10% ethanolic KOH agent, (b) BIT (bitter principles) after sprayed with vanillin sulpharic acid, (c) Fl (flavonoids) after sprayed with polythlene glycol, (d) saponins after sprayed with vanillin supharic acid), and(e) coumarins after sprayed with 10% ethanolic KOH. Abbreviations at the end of plates in each plateare: PL, *P. tomentosa L* leaves, PS*: P. tomentosa L* stems, EA*: E. ammak, EI: E. inerticulta, EC: E. cactus*

CONCLUSIONS

In conclusion, the methanolic extract of *E. cactus* Ehrenb and *E. ammak* Forssk showed a good antileishmanial activity with IC_{50} value>15.6 µg ml^{-1} relative to negative control. The preliminary phytochemical investigation reveals the extracts contain secondary metabolites that indicate these plants may be highly promising candidate drugs. Furthermore the results offer a scientific basis for the traditional use of investigated plants. This is a preliminary evaluation using promastigotes must be complemented with an evaluation using intracellular amastigotes in macro-phages. At the same time, an evaluation of the possible cytotoxicity of the tested plants is important. Further screening for Yemeni plants species especially that are used in traditional medicine must be done for searching of potential anti-leishmanial active constituents and record ethno-botanical data.

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AUTHOR'S CONTRIBUTION

This research work is part of A M. Sc thesis. **Al-Hajj MMA:** conducted experiments and wrote up the thesis. **Al-Shamahy HA:** supervised the experimental work, revised, and draft the manuscript. **Alkhatib BY:** co-advisor of the work. **Moharram BA** helped in chemical analysis. All authors revised the article and approved the final version.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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