CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *CALOTROPIS GIGANTEA* LEAF ESSENTIAL OIL

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Keywords: *Calotropis gigantea*, Essential oil; Phytol; Antimicrobial activity.

Essential oil from *Calotropis gigantea* (L) leaves was extracted by hydrodistillation method followed of solvent extraction and characterized by GC-FID and GC-MS techniques. The chemical profiling estimated the presence of 42 components, representing 82.5 % of the total oil composition. Phytol (17.94 %), phenylacetaldehyde (9.16 %), 4-methyl-1-heptanol (4.98 %), benzyl alcohol (4.10 %), 4-vinyl guaiacol (3.87 %), 4-methyl-3-penten-1-ol (3.83%), Gentanol (2.93 %), 2-hexyn-1-ol (2.86 %) and phenethyl alcohol (2.52 %) were found to be the major constituents. Further, biological activities of the extracted oil were studied on the fungal (*Candida albicans*) and bacterial (*Escherichia Coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*) pathogens. 100 µl essential oil extracted from leaf shows effective antimicrobial activity against selective bacterial and fungal pathogens. Among bacterial pathogens, leaf oil showed highest antimicrobial activity against *Pseudomonas aeruginosa* followed by *Escherichia coli* and *Staphylococcus aureus*.

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INTRODUCTION

Calotropis is a genus belonging to family Asclepiadaceae, comprised of six species of perennial shrubs distributed in tropical and subtropical Asia, and Africa.[1] Similar to *Calotropis procera*, *Calotropis gigantea* is found in the Indian subcontinent and have great economic importance.[2] The root bark is used to treat dysentery and elephantiasis, while the flowers in small doses give relief in colds, coughs, asthma, and indigestion.[1] The latex and roots are reported as indigenous medicine in birth control.[3] The bark can be used in fiber industries for manufacturing weave carpets, ropes, sewing thread and fishing nets.[4] Leaf extracts are used in agriculture to protect *Oryza sativa* from pathogenic fungus,[5] and in material science to synthesize nanomaterials.[6]

Interestingly, the plant is used as traditional biopesticides in controlling mollusks and mosquitoes.[7,8] Moreover, variety of chemical groups including cardenolides, steroids, flavonoids, terpenoids, cardiac glycosides, resins, fatty acids and non-protein amino acids etc. have also been identified in *C. gigantea*,[9] which leads to various applications in food, flavour and pharma industries to transformed into promising biologically active molecules for chemicals and drugs synthesis. For example, eugenol and guaiacol are important constituents of *Calotropis* essential oil, which is recently being explored as a substitute for fossil-based chemicals for making biobased materials and green chemicals.[10,11] Interestingly, Ashori et al. extensively studied different parts of *C. gigantea* in terms of chemical, morphological, and mechanical properties, and the results show that bark materials have comparatively higher cellulose content than the latex and flower[12].

Consequently, the bark and another woody part of this plant can be utilized and explored as a resource for green chemicals and biofuels as reported in previous literature.[13,14] With diverse chemical functionalities, essential oils (EO) are considered to be promising "green" alternative in the food, pharmaceutical, and agriculture industries[10,11,15] resulted to give effective antimicrobial, nematicidal, antiviral, insecticidal as well as antifungal properties.[16] EO constitutes about 20–60 components from fairly high concentrations (20–70 %) to traces. The products of higher concentrations (e.g. terpenes, terpenoids, aromatics, etc.) are responsible for a biological activities[17] while, minor components of EO shows synergism with major constituents.[15] Variety of plant species and their corresponding parts such as methanol extract of the root bark of *C. gigantea* is reported as potent antifeedant for the desert locust,[18] alcoholic root (100 mg kg^{-1} day^{-1}) and flower extracts are reported correspondingly for pregnancy interceptive and analgesic activities in the rats,[19] while the stem and flower extracts resulted in various chemical and biological activities.[9]

Though, the several kinds of the literature indicated the EO extraction and biological activity of extracts from *Calotropis* sp., very few are available on leaf extracts of EO of the *Calotropis gigantea* in particular. Nevertheless, no study is available on chemical profiling and antimicrobial activities of *C. gigantea*n leaf EO. Thus, in the present study, essential oil extraction of *C. gigantea* leaves and their chemical profiling, are investigated. In order to test biological activities, antimicrobial activities of EO against pathogenic fungal and bacterial species are studied. The chemical compositions of the essential oils were analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography with mass spectrometry (GC-MS). This study includes identification of about 42 compounds from the hydrodistilled extract of leaf from *C. gigantea* corresponding to 82.5 % of total oil.

MATERIALS AND METHODS

Source of the plant

Fresh leaves of *Calotropis gigantea* were collected during flowering season from the roadsides (April 2014), dry wasteland area adjacent to Muzaffarnagar (29.4723° N, 77.7089° E) having weather (29°C, Wind W at 6 km h^{-1}, 80 % humidity), Uttar Pradesh State, India. Collected plant materials were submitted for identification at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. A voucher specimen is available in the herbarium division of the NISCAIR.[20]

Isolation of essential oil

Leaves were washed properly three times with fresh water and dried in shade till constant weight (difference between two consecutive weight not more than 0.05 mg) in properly ventilated room under fan in ambient conditions (27 °C) to prevent loss of essential oil. Dried leaves were crushed, weighed and subjected to hydro-distillation in 5 batches of 170 g each in 600 mL distilled water in Clevenger type apparatus for 4-5 h. The distillated product was further extracted with n-hexane and passed through anhydrous sodium sulphate to remove residual water. The product was concentrated by evaporating hexane under reduced pressure. Total oil obtained was 0.95 g. The yield of essential oil obtained was found to be 0.11 % (w/w).

Essential oil composition analysis by gas-chromatography (GC)/mass spectrometry (MS)

Essential oil composition of *Calotropis gigantea* was analyzed using Shimadzu GC-2010 equipped with flame ionization detector using Rtx-5MS (30 m x 0.25 mm ID x 0.25 μm) column. Nitrogen was used as carrier gas at 234.6 kPa inlet pressure. Oven temperature was programmed from 50 ºC for 3.0 minutes, 3.0 ºC min^{-1} to 200 ºC for 2.0 minutes, 10 ºC min^{-1} to 280 ºC for 7 minutes. The injector and detector temperature were 250 ºC and 260 ºC respectively. The oil was injected neat with split ratio of 10. Relative amount of individual components are based on GC peak area percentage obtained without FID response factor correction. The retention Indices were obtained from GC by logarithmic interpolation between bracketing n-alkanes. The homogenous series of n-alkanes (C_8-C_{22}, Polyscience, Niles, USA) were used as standard.

GC-MS data were obtained on Shimadzu GCMS-QP2010 plus using same chromatographic conditions and column used for GC-FID i.e. Rtx-5MS (30 m x 0.25 mm ID x 0.25 μm) column and helium as carrier gas. Temperature programming was 50 ºC for 3.0 minutes, 3.0 ºC min^{-1} to 200 ºC for 2.0 minutes, 10 ºC min^{-1} to 280 ºC for 7 minutes.

Identification of constituents

The individual peaks in GC of *Calotropis gigantea* leaf oil were identified by comparison of their retention indices on the SP Rtx-5MS (30 m x 0.25 mm ID x 0.25um) column with literature values[5] and were reported based on its area % response by GC-FID. The essential oil constituents were confirmed by matching of mass spectra of peaks with FFNSC2, NIST08 and Wiley8 library. Identification was done by GC-MS while RI and quantification done by GC-FID.

Antimicrobial activity

Microbial strains used

The antimicrobial activity of essential oil was performed using the following microbial stains procured from the Institute of Microbial Technology, Chandigarh, India. Details of the bacterial and fungal cultures used in the study are given in the Table 1.

Table 1. Lists of microbial stains and their corresponding medium

Organism	Medium	Incubation	
		***T*, °C**	***t*, h**
Escherichia coli ATCC8739	Soyabean-casein digest agar; Soyabean-casein digest broth	37°± 1	24 hours
Pseudomonas aeruginosa ATCC9027	Soyabean-casein digest agar; Soyabean-casein digest broth	37°± 1° C	24 hours
Staphylococcus aureus ATCC6538	Soyabean-casein digest agar; Soyabean-casein digest broth	37°± 1° C	24 hours
Candida albicans ATCC10239	Sabouraud dextrose agar; sabouraud dextrose broth	25°± 1° C	48 hours

Inoculum preparation

Working cultures were prepared from the glycerol culture stocks maintained in the laboratory. A loopful of individual culture from agar slant was inoculated in to the broth medium aseptically and incubated as per the temperature and time combination mentioned in the Table 1. Cultures grown in the broth medium were harvested by centrifugation, washed and resuspended in sterile saline solution (0.85 %) to obtain a culture concentration in the range of 1x 10^5 and 1 x 10^6 cfu mL^{-1}. Culture concentrations were standardised turbidometrically.

Antimicrobial activity

Antibacterial activity by well diffusion method:

The essential oil obtained from hydro-distillation of shade dried leaf was screened for their antibacterial activity in vitro by well diffusion method. Lawn culture was used using the test organism on Soyabean-casein digest agar in triple section Petri plate. The inoculated plates were kept aside for

few minutes, and using well cutter, wells were made in center of each section under aseptic condition. A fixed volume 100 µl of *Calotropis gigantea* leaf essential oil was then introduced into the well. The plate with bacteria was incubated at 37 ˚C for 24 h. The activity of essential oil was determined by measuring the diameter of zone of inhibition.

Antifungal activity by well diffusion method:

The essential oil obtained from hydrodistillation of shade dried leaves was also screened for antifungal activity in vitro using well diffusion method. Lawn culture was used using the test organism on Sabouraud dextrose agar in triple section plate. The inoculated plates were kept aside for few minutes, and using well cutter, wells were made in center of each section under aseptic condition. A fixed volume 100 µl of *C. gigantea* leaf essential oil was then introduced into the well. The plate of fungi was incubated at room temperature for 48 h. The activity of essential oil was determined by measuring diameter of zone of inhibition.

RESULTS AND DISCUSSION

Chemical investigation of essential oil of traditionally useful *Calotropis gigantea* which is reported for the first time in the literature and total ion chromatogram (TIC) of this oil showed 42 compounds corresponding to 82.5 % total area percentage. The yield of essential oil obtained by hydro distillation from *C. gigantea* was 0.11 % (w/w) with respect to total dry mass of the leaves. In order to explore useful products of C. *gigantea*, significant contributions have been made to identify phytochemical constituents and important activities[2,9,19] studied in different parts of the plant except for the leaves. In addition to the beneficial effect of latex,[3,7,8] root, and flowers,[1,2,9] Kumar et al. recently studied aqueous leaf extract of C. *gigantea* for antibacterial activity and found effective against *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Micrococcus luteus* and *Klebsiella pneumonia* species.[22] Motivated from this study, the extraction and identification of chemical composition of C. *gigantea* leaf essential oil was performed and analyzed on GC-FID and GC-MS for further structural confirmation under similar chromatographic conditions as mentioned in the material method section. The chemical profiling of the EO reveals the dominance of oxygenated diterpenes (phytol), aromatic alcohol (benzyl alcohol) and linear chain alcohol (4-methyl-1-heptanol) as shown in the Table-2 (Supplementary Information). Among major constituents of leaf essential oil, the main peaks were dominated with phytol (17.94 %), phenylacetaldehyde (9.16 %), 4-methyl-1-heptanol (4.98 %), benzyl alcohol (4.10 %), 4-vinyl guaiacol (3.87 %), 4-methyl-3-penten-1-ol (3.83 %), gentanol (2.93 %), 2-hexyn-1-ol (2.86 %) and phenethyl alcohol (2.52 %).

The result of *C. gigantea* leaf is in good agreement with the result of Schmelzer et al. on another species (*Gongronema lantifilium*) of the same family *Asclepiadaceae* where phytol was measured to be 15.5% along with 19.5 % linalool. The compositional changes may be due to the geographical areas, a method of extraction as well as species of the plant. For example, the essential oil extracted from *Calotropis* procera from Nigeria is reported to be 33.6 % phytol while the same species from Iran shows about 5 % more phytol content (38.2%).[23,24]

Phytol, phenylacetaldehyde and 4-vinylguaiacol are an important fragrant ingredient used in many house-hold products including cosmetics, fine fragrances, shampoos, toilet soaps and detergents.[25,26,27] In addition to that, the derivatives of phytol show crucial pharmacological effects in humans and other animals,[25] while 4-vinylguaiacol can act as a green source to produce acetovanilline and ethyl guaiacol (used in perfumery) as well as biodegradable polystyrene.[27]

The phytochemical analysis of leaf EO was investigated using nonpolar solvent, n-hexane. Nonpolar solvents are mostly used for defatting the dried plant material.[29] During the chromatographic analysis of EO, the compound eluted in order of their molecular weight, boiling point, and chemical structures. The molecule with low boiling point eluted first followed by the increasing boiling point/molecular weight of the molecule. As a result, phytol with higher molecular weight (296.53 g mol^{-1}) eluted at the end (Table 2, Fig 1). Linear chain and the aromatic compound have a chain in their structure. Gentanol (116.2 g mol^{-1}), for example, is a linear chain alcohol with seven carbon chain eluted before benzyl alcohol (108.14 g mol^{-1}). This could be due to its interaction with the stationary phase column. Similarly, 4-methyl-3-penten-1-ol (100.16 g mol^{-1}) eluted before 2-hexyn-1-ol (98.15 g mol^{-1}) since it has single covalent bond whereas 2-hexyn-1-ol has a triple bond.

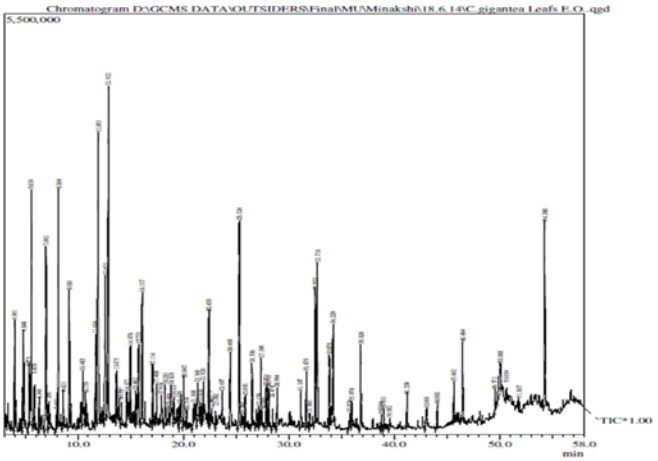
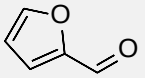
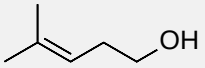
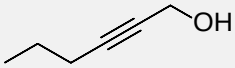
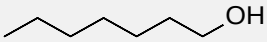
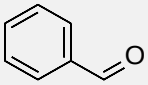
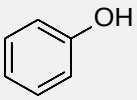
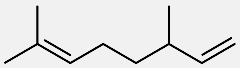
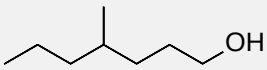
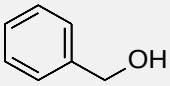
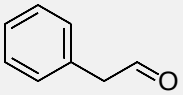
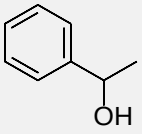
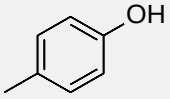
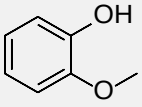


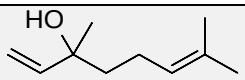
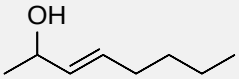
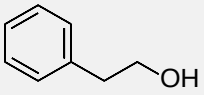
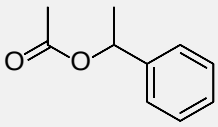
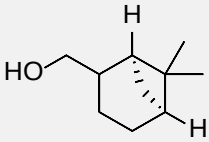
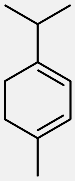
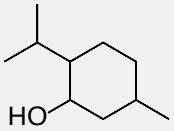
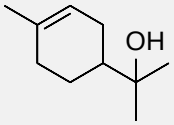
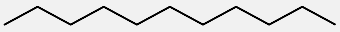
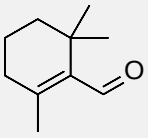
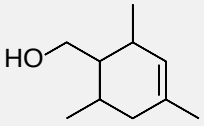
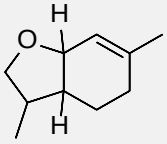
Figure 1. GC/MS spectra of essential oil from leaf of *Calotropis gigantea*

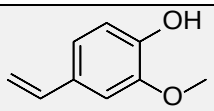
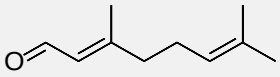
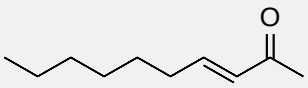
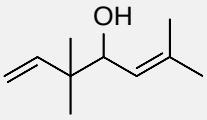
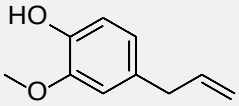
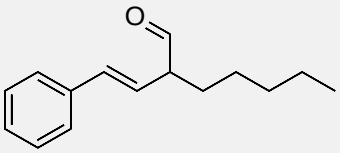
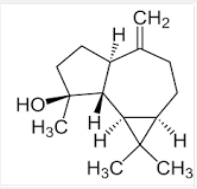
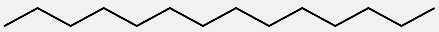
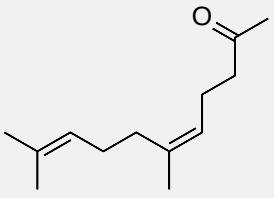
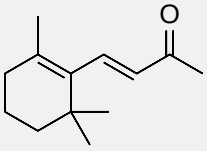
Moreover, a number of other odor-active compounds such as ±linalool (0.49 %), hexanol (0.48 %), α-citral (0.06 %), eugenol (0.30 %) etc. are identified in this study. These minor products are used extensively in chemical industries as perfumery or precursors for making value-added products along with some pharmaceutical purposes.[28,11,29] α-Citral, (±)-linalool and eugenol can also show biological activities against pathogenic fungus. In addition, compounds containing strong antimicrobial activity like benzyl alcohol (4.10%), (+)-β-citronellene (1.39 %), p-cresol (1.07 %), and guaiacol (0.86 %) have also been identified in the leaf

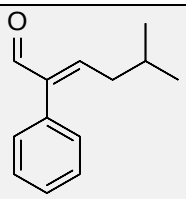
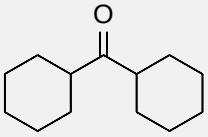
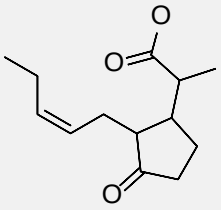
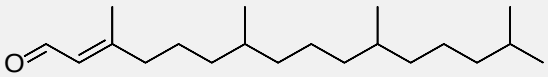
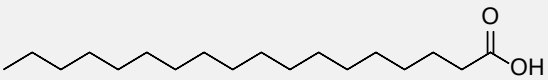
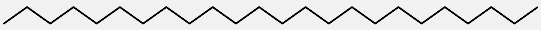
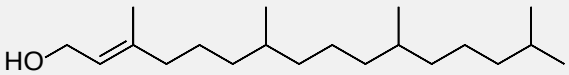
essential oil. These products can also show repellent properties against some insects, and thereby being used extensively as an important ingredient in insecticide formulation.

Table 2. Major identified products of Essential oil from *Calotropis gigantea* Leaf EO as measured by GC-FID[a]

Peak#	R.Time	Area	Area%	RI	Structure and Name	M/Z
1	4.848	431538	2.4992	1026	 2-Furancarboxaldehyde	96
2	5.619	661265	3.8296	1058	 4-Methyl-3-penten-1-ol	100
3	7.002	494423	2.8634	1112	 2-Hexyn-1-ol	98
4	8.164	506301	2.9322	1148	 Gentanol	116
5	9.183	324934	1.8818	1180	 Benzaldehyde	106
6	10.483	273777	1.5855	1214	 Phenol	94
7	11.694	241298	1.3974	1225	 β-Citronellene	138
8	11.953	859671	4.9787	1251	 4-Methyl-1-Heptanol	130
9	12.625	708290	4.102	1258	 Benzyl alcohol	108
10	12.922	1582376	9.1641	1266	 Phenyl acetaldehyde	120
11	13.675	114682	0.6642	1276	 alpha-Methylbenzyl alcohol	122
12	14.671	185932	1.0768	1304	 p-Cresol	108
13	14.976	148515	0.8601	1310	 Guaiacol	124

14	15.462	85003	0.4923	1316	 Linalool	154
15	15.77	215671	1.249	1329	 Oct-3-en-2-ol	128
16	16.117	435391	2.5215	1337	 Phenethyl alcohol	122
17	17.114	109729	0.6355	1350	 Gardenol	164
18	17.918	125137	0.7247	1367	 cis-Myrtanol	154
19	18.283	144493	0.8368	1392	 α-Terpinene	136
20	18.828	49071	0.2842	1413	 Menthol	156
21	19.658	110346	0.6391	1431	 Alpha-Terpineol	154
22	20.047	285531	1.6536	1436	 n-Undecane	156
23	21.008	95278	0.5518	1457	 β-Cyclocitral	152
24	21.779	43108	0.2497	1492	 Isocyclogeraniol	154
25	23.697	79634	0.4612	1529	 Dill ether	152

26	25.324	667795	3.8674	1546	 4-Vinylguaiacol	150
27	25.881	10643	0.0616	1583	 α-Citral	152
28	26.536	83596	0.4841	1606	 Heptylidene acetone	154
29	26.957	199462	1.1552	1613	 Artemisia alcohol	154
30	27.179	52433	0.3037	1621	 Eugenol	164
31	27.873	166014	0.9614	1676	 Z-Amyl cinnamaldehyde	216
32	28.474	67332	0.3899	1692	 Spathulenol	220
33	28.904	52044	0.3014	1704	 n- Tetradecane	198
34	31.147	90802	0.5259	1768	 Nerylacetone	194
35	32.522	316913	1.8354	1808	 β-Ionone	192

36	32.718	426348	2.4691	1814	 Hex-2-enal (5-methyl-, 2-phenyl-)	188
37	33.87	167676	0.9711	1848	 Cyclohexyl ketone	194
38	38.794	66462	0.3849	1939	 Methyl-Jasmonate	224
39	45.662	51359	0.2974	2228	 Phytone	268
40	50.088	116506	0.6747	2362	 Stearic acid	284
41	50.654	122057	0.7069	2378	 n-Tetracosane	338
42	54.28	3097846	17.9408	2659	 Phytol	296

where RT = Retention time, RI = Retention index, [a] List of products as confirmed by GC-MS

Since the plant is reported effectively against fungal and bacterial infections such as leprosy, tuberculosis and lupus, hence there is a need to investigate products that can serve as an effective antimicrobial and anti-biotic agent. In order to test these properties in leaf extracts of C. *gigantea* EO, a fixed volume 100 µl of leaf EO was used by well plate method against *Candida albicans*, a fungal pathogen as well as bacterial pathogens such as *Pseudomonas aeruginosa*, *Escherichia Coli*, and *Staphylococcus aureus* respectively. The activities of EO was determined by measuring zone of inhibition (mm). The results show that extracted EO gave promising biological activities and thereby exhibit strong inhibitory effect (5mm) against fungal pathogen, *Candida albicans* as presented in Table 3.

Table 3. Antifungal activity of essential oil from leaves of *Calotropis gigantea*

Microbial Strains	Fungus/Bacteria	ZOI (mm)
Candida albicans	fungus	5
Staphylococcus aureus	bacterium	4
Escheriachia coli	bacterium	5
Pseudomonas areuginosa	bacterium	7

ZOI, zone of inhibition

This is probably due to high benzyl alcohol (+)-β-citronellene, p-cresol, and guaiacol content. Similarly, the oil shows good activity against bacteria *Pseudomonas aeruginosa* followed by *Escherichia Coli* and *Staphylococcus aureus* which cover 7 mm, 5 mm and 4 mm zone of inhibition (ZOI) respectively.

CONCLUSION

This study reports EO extraction from *C. gigantea* leaf, their compositional analysis and tests for biological activity for the first time to the best of our knowledge, and a high phytol content 17.94 % was obtained in the oil. In addition, phenylacetaldehyde (9.16 %), 4-methyl-1-heptanol (4.98 %), benzyl alcohol (4.10 %), 4-vinylguaiacol (3.87 %), 4-methyl-3-penten-1-ol (3.83 %), gentanol (2.93 %), 2-hexyn-1-ol (2.86 %) and phenethyl alcohol (2.52 %). The biological activities of EO were tested against *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* and showed promising results. Due to the presence of diverse functionality, the EO or isolated molecules can be applied as a natural preservative, fuel additives, and drugs including pesticides and bulk chemicals.

eugenol and guaiacol are important building block chemicals in the range of traditionally produced molecules from fossil resources. The production/isolation of these chemicals from *Calotropis gigantea* could help in exploring biobased chemicals production to fulfill future energy and chemical demands.

ACKNOWLEDGMENTS

Authors acknowledge Dr. Imteyaz Alam of Department of Chemical Engineering, Institute of Technology (IIT) Delhi, Dr. Ovais Rizvi and Dr. Pooja Sharma of Department of Chemistry, Jamia Hamdard for their continuous support and scientific discussions.

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Received: 15.11.2018.
Accepted: 13.12.2018.

BIOLOGICAL ACTIVITY OF SOME NEWLY SYNTHESIZED HYDRAZONE DERIVATIVES DERIVED FROM (DICYCLOPROPYLMETHYLENE)HYDRAZONE

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Keywords: Hydrazones; Dicyclopropyl ketone; Anticancer activity; Antibacterial activity.

A series of new hydrazone derivatives were synthesized using dicyclopropyl ketone as starting material. Unexpected reaction of salicylaldehyde with (dicyclopropylmethylene)hydrazine yielded the appropriate bis(salicylaldehyde azine). Treatment of the (dicyclopropylmethylene)hydrazine with the appropriate coupling agents gave the corresponding imides, imidazolones, oxazolones, quinazolinones and triazoles, respectively. The synthesized compounds were characterized using IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data. These newly formed products were tested for their antibacterial and antifungal activities and most of the compounds showed high activity compared with Ciprofloxacin as positive controls. In addition, the compounds were also, examined for their anticancer activities against breast cancer cell line (MCF7) compared with cisplatin as a positive control. The novel synthesized compounds showed satisfactory activity against (MCF7) with mean IC_{50} values ranging from 21.5 to 100 μM.

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Introduction

Hydrazones have diverse interesting biological activities[1] including antitumor,[2–4] anti-inflammatory,[5] antimalarial,[6] antimicrobial,[7,8] insecticidal,[9] antiplatelet,[10] antibacterial, antifungal, antimycobacterial, cytotoxic and cytostatic activities.[11,12] Cu(II) phenanthroline hydrazone complexes are able to cleave DNA and show cytotoxic activity against cancer cells.[13] Aminoguanidine hydrazones (AGH's) have potential important towards developing focused adjuvants for antibiotic drug therapies against bacterial multidrug resistance.[14] These compounds also evaluated for their in vitro α-glucosidase inhibitory activity.[15] Hydrazones have been used to design materials that are used for pH-responsive drug delivery systems and these hydrazones were characterized by different spectral methods. Therefore there is scope to utilize these dendrimers to form hydrazone conjugates with the drugs (anticancer as well as antimicrobial) for more effective and targeted delivery in acidic environments.[16] Hydrazones have an attractive therapeutic approach, which is widely applied in the treatment of many diseases. For example, Alzheimer's disease (AD).[17] Moreover, the significance of hydrazones is due to their ability to extensively used as intermediates for the synthesis of heterocyclic compounds of pharmaceutical interests[18,19] and as transition metal chelators.[20,21] Therefore, and in view of these findings we report here in the utilization of (dicyclopropylmethylene)hydrazone (2) which has previously been prepared by the reaction of dicyclopropyl ketone and hydrazine hydrate [22] as a primitive compound for synthesis of novel of a series of new heterocyclic compounds with a biologically active pharmacophore (=NH–N-CH–) followed by screened for their antibacterial, antifungal and anticancer activities against breast cancer cell line (MCF7).

Experimental

All compounds were used without any purification or treatment. Reagents were used synthesized according to published literature. Thin-layer chromatography (TLC) was used to monitor the reaction and emphasizes the purity of the products. Melting points are recorded in Gallenkamp electric melting point apparatus and are uncorrected. The IR spectra $\acute{\upsilon}$ (cm^{-1}) were measured on FT-IR spectrophotometer in potassium bromide (KBr). The ^{1}H-NMR and ^{13}C-NMR spectra were measured were measured in DMSO-d6 as solvent at 500 MHz on a JNM-ECA500II NMR spectrometer using tetramethylsilane (TMS) as an internal reference and chemical shifts are expressed as δ ppm. The mass spectra (EI) were recorded on 70 eV with Kratos MS equipment.

Synthesis of (dicyclopropylmethylene)hydrazone (2)

Hydrazine hydrate (1 mmol) was added to an equimolar amount of a solution of dicyclopropyl ketone (1 mmol) in 15 mL of absolute ethanol. The reaction mixture was heated under reflux for 5 h, evaporate the excess ethanol and then left to cool down to 0°c. The solid product was obtained by pouring onto ice-water with stirring, filtered off, dried, the product is sufficiently pure and it didn't need purification to give 2 as Colorless powder; yield 92 %; m.p. 85° C. IR (KBr, ν/cm^{-1}): 3425, 3395 (NH_2), 1604 (C=N).^{1}H NMR (DMSO-d6) δ ppm 0.6-1.07 (m, 8H, $4CH_2$), 2.25 (m, 4H, $2CH,NH_2$). ^{13}C-NMR (DMSO-d6): δ ppm 5.6, 7.24, 9.36, 11.45 and 165.8. MS (EI): (m/z, %), 124 (M^{+}-1, 1), 108 (100).

General procedure for the synthesis of 3-10

A mixture of equimolar amount of **2** (1 mmol) and appropriate aldehyde (p-methoxybenzaldehyde, p-nitrobenzaldehyde, furfural, isatin or salicylaldehyde) (1

mmol) in absolute ethanol (30 ml) was refluxed in water bath for 6-8 h. the reaction mixture was left to cool, the resulting precipitate was filtered off and recrystallized from ethanol to give **3-8**.

1-(Dicyclopropylmethylene)-2-(4-methoxybenzylidene)-hydrazine (3a)

Yellow needles; yield 78 %; mp 164-6 °C IR (KBr, ν/cm^{-1}): 2967-2840 (CH aliph.), 1659, 1600 (2C=N).^{1}H NMR (DMSO-d6) δ ppm 0.1-1.06 (m, 10H, H-aliph), 3.79 (s, 3H, OCH_3) and 7.035-7.8 (m, 4H, H-Ar) 8.6 (s, 1H, CH=N).^{13}C-NMR (DMSO-d6): δ ppm 5.6, 7.24, 9.36, 11.45 and 165.8. MS (EI): (m/z, %), 241 (M^+-1, 9.08), 160 (100).

1-(Dicyclopropylmethylene)-2-(4-nitrobenzylidene)hydrazine (3b)

Yellow crystals; yield 92 %; mp. >250 °C. IR (KBr, ν/cm^{-1}): 2961-2830 (CH aliph.), 1609 (2C=N). ^{1}H NMR (DMSO-d6) δ ppm 0.12-1.4 (m, 10H, H-aliph), 6.8-7.1 (m, 4H, H-Ar) and 8.3 (s, 1H, CH=N). MS (EI): (m/z, %), 257 (M^+, 23).

1-(Dicyclopropylmethylene)-2-(furan-2-ylmethylene)hydrazine (3c)

Bale yellow needles; yield 81 %; mp 104 °C, IR (KBr, ν/cm^{-1}): 2927-2850 (CH aliph.), 1656-1651 (2C=N).^{1}H NMR (DMSO-d6) δ ppm 0.1-1.6 (m, 10H, H-aliph), 6.5-7.5 (m, 3H, H-Ar) and 8.1 (s, 1H, CH=N). MS (EI): (m/z, %), 202 (M^+, 60)

3-((Dicyclopropylmethylene)hydrazono)indolin-2-one (4)

Dark red crystals; yield 88 %; mp >300 °C. the reaction mixture with few drops of acetic acid as catalyst. IR (KBr, ν/cm^{-1}):3277(NH), 1723, 1613 (C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 0.9-1.3 (m, 10H, H-aliph), 6.8-7.5 (m, 4H, H-Ar) and 10.9(s, 1H, NH). ^{13}C-NMR (DMSO-d6): δ ppm 7.36, 9.2, 13.13, 110.6, 111.12, 122.05, 127.8, 128.2, 134.4, 144.73, 145.19 and 163.4 MS (EI): (m/z, %), 253 (M^+, 100).

Ethyl-3-((dicyclopropylmethylene)hydrazono)butanoate (5)

Yellow crystals; yield 68 %; mp190 °C. IR (KBr, ν/cm^{-1}):1683, 1613 (C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 0.9-1.0 (m, 10H, H-aliph), 1.1(t, 3H, CH_3), 2.2(S, 3H, CH_3), 1.1(t, 3H, CH_3), 2.2(S, 2H, CH_2)and 4.0(q, 2H, CH_2 ester). ^{13}C-NMR (DMSO-d6): δ ppm 12.8, 14.3, 17.7, 58.7, 103.8, 11.9, 138.6, 149.8, 159.7, and 166.8 MS (EI): (m/z, %), 236 (M^+, 2.5).

2,2'-(Hydrazine-1,2-diylidenebis(methanylylidene))-diphenol(10)

Bright yellow crystals ; yield 95 %; mp 205-7 °C

IR (KBr, ν/cm^{-1}):3445(OH), 1622 (2 C=N).^{1}H NMR (DMSO-d6) δ ppm 6.9-7.6(m, 8H, H-Ar), 8.9(s, 2H, CH=N) and 11.1(s, 2H, OH). MS (EI): (m/z, %), 240 (M^+, 17) and 185(100).

General procedure for the synthesis of 7a, 8a

Thiosemicarbazide (1 mmol) was added to an equimolar amounts of solution of **3a** (1 mmol) in 25 mL of absolute ethanol and few drops of acetic acid. The reaction mixture was heated under reflux for 12 h then the precipitated crude product was filtered off.

4-((Dicyclopropylmethylene)amino)-5-(4-methoxyphenyl)-4,5-dihydro-1H-1,2,4-triazol-3-amine(7a)

White crystals (from ethanol) 62 %, m.p 160-162 °C. IR (KBr, ν/cm^{-1}): 1793,1725, 1653 (C=O,2 C=N).^{1}H NMR (DMSO-d6) δ ppm 1.02 1.4 (m, 10H, H-aliph) and 3.8(s, 3H, OCH_3), 5.8 (s, 1H, CH), 6.7, 7.8 (m, 4H, H-Ar), 8.6 (s, 2H, NH_2) and 9.8 (s, 1H, NH). MS (EI): (m/z, %), 268 (M^+-OCH_3, 6.6).

5'-Amino-4'-((dicyclopropylmethylene)amino)-2',4'-dihydro-spiro[indoline-3,3'-[1,2,4]triazol]-2-one (8a)

Yellow crystals (from ethanol) 75 %, m.p 238-240 °C IR (KBr, ν/cm^{-1}): 3419, 3351(2NH), 3259, 3190(NH_2), 1679, 1613 (C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 1.0-1.055 (m, 10H, H-aliph), 6.8-7.3 (m, 4H, H-Ar), 10.5 (s, 1H, NH), 10.6 (s, 1H, NH) and 11.1 (s, 2h, NH_2) .^{13}C-NMR (DMSO-d6): δ ppm 10-20, 109.9, 111.03, 117.4, 119.98,162.7, 178.66 and 181.13 MS (EI): (m/z, %), 313 (M^++2, 8.4).

General procedure for the synthesis of 7b, 8b

A mixture of **4** (1 mmol) and of benzohydrazide (1 mmol) in 20 ml absolute ethanol in the presence of alcoholic potassium hydroxide (1.5 mmol) was refluxed for 22 h. The reaction mixture was cooled, then poured into ice-water, then it was acidified with diluted HCl to pH 6. The formed crude product was filtered off,

1,1-Dicyclopropyl-N-(5-(4-methoxyphenyl)-3-phenyl-1,5-dihydro-4H-1,2,4-triazol-4-yl)methanimine (7b)

Brown crystals (from ethanol) 62 %, m.p >250 °C IR (KBr, ν/cm^{-1}): 3419, 3351(2NH), 3259, 3190(NH_2), 1679, 1613 (C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 1.0-1.055 (m, 10H, H-aliph), 6.8-7.3 (m, 4H, H-Ar), 10.5 (s, 1H, NH), 10.6 (s, 1H, NH) and 11.1 (s, 2h, NH_2) .^{13}C-NMR (DMSO-d6): δ ppm 10-20, 109.9, 111.03, 117.4, 119.98,162.7, 178.66 and 181.13 MS (EI): (m/z, %), 313 (M^++2, 8.4)

4'-((Dicyclopropylmethylene)amino)-5'-phenyl-2',4'-dihydro-spiro[indoline-3,3'-[1,2,4]triazol]-2-one (8b)

Yellow crystals (from ethanol) 62 %, m.p 262-264 °C. IR (KBr, ν/cm^{-1}): 3349 (NH), 1685, 1601 (2C=N).^{1}H NMR (DMSO-d6) δ ppm 0.9-2.1 (m, 10H, H-aliph), 3.8 (s, 3H, CH_3) and 7-7.9 (m, 9H, H-Ar) MS (EI): (m/z, %) 360 (M^+- 1, 15.1).

Synthesis 2-(3-amino-4-((2-hydroxybenzylidene)amino)-4,5-dihydro-1H-1,2,4- triazol-5-yl)phenol (11a)

Thiosemicarbazide (1 mmol) was added to equimolar amounts of the solution of **10** (1 mmol) in 25 mL of absolute

ethanol and few drops of acetic acid. The reaction mixture was heated under reflux for16 h, the precipitated of the crude product was filtered and further purified by recrystallization from methanol to give **11a** as faint yellow crystals; yield 95 %; mp 226-8 ºC. IR (KBr, ν/cm^{-1}):3443, 3320(NH_2),3173, 3137 (2CH=N), 1723, 1613 (C=N).^{1}H NMR (DMSO-d6) δ ppm 6.7-8.0 (m, 8H, H-Ar), 8.3 (s, 1H, CH=N),9.8(s, 1H, NH_2)and 11.3(s, 1H, OH). ^{13}C-NMR (DMSO-d6): δ ppm 116, 119.34, 120.37, 126.7, 131.17, 139.7, 156.4 and 177.6 MS (EI): (m/z, %), 297 (M^+, 2.5), 195(100).

Synthesis of 2-(4-((2-hydroxybenzylidene)amino)-3-phenyl-4,5-dihydro-1H-1,2,4-triazol-5-yl)phenol(11b)

A mixture of **10** (1mmol) and (1 mmol) of benzohydrazide in 20 ml absolute ethanol in the presence of alcoholic potassium hydroxide (1.5 mmol) was refluxed for 8 h. The reaction mixture was cooled, then poured into ice-water, then it was acidified with dilute HCl to pH 6. The formed crude product was filtered off, dried, and crystallized from methanol to give (55 %) of **11b** as yellow crystals; yield 54 %; mp. 198-200 ºC.

IR (KBr, ν/cm^{-1}): 1689,1623 (2 C=N).^{1}H NMR (DMSO-d6) δ ppm 6.5-7.1(m, 8H, H-Ar), 8.5(s, H, CH=N) and 11.1(s, 2H, 2OH). MS (EI): (m/z, %), 356 (M^+, 20).

General procedure for the synthesis of 12, 13

A mixture of dicyclopropyl ketone **1** (1 mmol) and cyanoacetic acid hydrazide (1 mmol) in 25 ml absolute ethanol was refluxed for 3 h on a water bath. After leaving the mixture to cool to room temperature, the white precipitate that formed was filtered off to be the crude product of **12**. The filtrate was cooled to 0ºc the brown pellets that precipitated was filtered off to afford **13.**

2-Cyano-N'-(dicyclopropylmethylene)acetohydrazide (12)

White crystals (from ethanol) 65 %, m.p 125-126 ºC. IR (KBr, ν/cm^{-1}):3198 (NH), 2264 (cyano), 1679 (C=N).^{1}H NMR (DMSO-d6) δ ppm 0.6-2.03 (m, 10H, H-aliph), 3.9 (s, 2H, CH_2) and 10.86(s, 1H, NH). ^{13}C-NMR (DMSO-d6): δ ppm 5.92, 6.86, 10.5, 11.03, 24.57, 116.24, 157.92 and 165.03 MS (EI): (m/z, %), 191 (M^+, 13), 123(100).

5-Amino-1-(dicyclopropylmethyl)-1,2-dihydro-3H-pyrazol-3-one (13)

Brown pellets (from ethanol) 33 %, m.p 210 °C. IR (KBr, ν/cm^{-1}):3337(NH), 3166, 3100 (NH_2), 1689, 1654 (C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 0.8-1.8 (m, 10H, H-aliph), 4.1(s, 1H, CH-N), 5.6 (s, 1H, NH), 5.8 (S, 1H, H-Ar) and 9.8(s, 2H, NH_2). ^{13}C-NMR (DMSO-d6): δ ppm 18.6, 36.27, 56.08, 74.23, 157.0, 159.3 and 171.7. MS (EI): (m/z, %), 193 (M^+, 1.5), 69(100).

Synthesis of 1-(dicyclopropylmethylene)-3-(2-methyl-4-oxoquinazolin-3(4H)-yl)thiourea (15)

A mixture of equimolar amount of dicyclopropyl ketone (1 mmol) and thiourea derivative **14** (1 mmol) in absolute ethanol (25 ml) was refluxed in water bath for 12 hr. the reaction mixture was left to cool, the resulting precipitate was Filtered off and recrystallized from ethanol to give **15** as brown needles in 45 % yield, mp 135-2 °C. IR (KBr, ν/cm^{-1}):3432 (NH), 1675, 1660 (C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 1.1-1.8 (m, 13H, H-aliph, CH_3), 6.4-7.3 (m, 4H, H-Ar) and 8.2 (s, 1H, NH). MS (EI): (m/z, %), 326 (M^+, 100).

General procedure for the synthesis of 16-20

To a solution of **2** (5 mmol) and 25 ml of glacial acetic acid, (5 mmol) of phthalic anhydride, maleic anhydride, succinic anhydride, 4-benzylidene-2-methyloxazol-5(4H)-one, 4-benzylidene-2-phenyloxazol-5(4H)-one or 2-methylbenzoxazinone was added. The reaction mixture was refluxed for 6-24 h. The solution was poured on ice-cold water. The formed precipitate was filtered, washed with water, dried and crystallized from the appropriate solvent.

2-(2-(Dicyclopropylmethylene)hydrazine-1-carbonyl)benzoic acid (16)

White crystals (from acetic acid) 72 %, m.p >250 °C. IR (KBr, ν/cm^{-1}):3459(OH), 1746, 1660, 1605 (2C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 0.6-1.8 (m, 10H, H-aliph), 7.8-8.1 (m, 5H, H-Ar,NH) and 11.5(s, 1H, COOH). ^{13}C-NMR (DMSO-d6): δ ppm 18.6-74.23, 124.8, 125.14, 127.16, 128.8, 132.6, 136.2 and 163.44. MS (EI): (m/z, %), 272 (M^+, 25).

1-((Dicyclopropylmethylene)amino)-1H-pyrrole-2,5-dione (17)

Brown crystals (from methanol) 55 %, m.p >260 °C. IR (KBr, ν/cm^{-1}): 1739, 1634 (2C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 0.8-2.1 (m, 10H, H-aliph), 6.2 (dd, 2H, H-Ar). ^{13}C-NMR (DMSO-d6): δ ppm 38.9, 20.4, 20.6, 21.08, 29.04, 131.65, 166.85 and 172.06. MS (EI): (m/z, %), 204 (M^+, 56).

1-((Dicyclopropylmethylene)amino)pyrrolidine-2,5-dione (18)

White needles (from DMF) 82 %, m.p 245-8 ºC IR (KBr, ν/cm^{-1}): 1735 (2C=O).^{1}H NMR (DMSO-d6) δ ppm 1.8-3.5 (m, 14H, H-aliph). ^{13}C-NMR (DMSO-d6): δ ppm 26.3, 172.0 and 172.2. MS (EI): (m/z, %), 206 (M^+, 62).

5-Benzylidene-3-((dicyclopropylmethylene)amino)-2-methyl-3,5-dihydro-4H-imidazol-4-one(19a)

Brown crystals (from ethanol) 62 %, m.p 152-154 °C. IR (KBr, ν/cm^{-1}): 1704, 1645, 1602 (C=O, 2C=N).^{1}H NMR (DMSO-d6) δ ppm 1.9-2.0 (m, 13H, H-aliph, CH_3) and 6.9-8.3 (m, 6H, H-Ar). ^{13}C-NMR (DMSO-d6): δ ppm 10.0, 14.04, 20.50, 21.06, 112.2, 130.4, 132.4, 136.9, 141.15, 159.34, 169.12 and 172.03. MS (EI): (m/z, %), 289 (M^+- 4, 10.6).

5-Benzylidene-3-((dicyclopropylmethylene)amino)-2-phenyl-3,5-dihydro-4H-imidazol-4-one (19b)

Yellow crystals (from acetic acid) 74 %, m.p 158-160 °C. IR (KBr, ν/cm^{-1}): 1793,1725, 1653 (C=O,2 C=N).^{1}H NMR (DMSO-d6) δ ppm 0.8-2.1 (m, 10H, H-aliph) and 7.4-8.3(m, 11H, H-Ar,CH=C).^{13}C-NMR (DMSO-d6): δ ppm 10-20,

125.1-133.7 Ar-C, 163.06 and 166.9 MS (EI): (m/z, %), 352 (M^+, 1.9).

3-((Dicyclopropylmethylene)amino)-2-methylquinazolin-4(3H)-one (20)

Faint yellow needles (from ethanol) 82 %, m.p 230-232 °C IR (KBr, ν/cm^{-1}): 1675, 1660 (C=O, C=N).^{1}H NMR (DMSO-d6) δ ppm 1.02-2.1 (m, 13H, H-aliph, CH_3) and 7.9-8.2 (m, 4H, H-Ar). MS (EI): (m/z, %), 267 (M^+, 3.6).

Synthesis of 2,2-dicyclopropyl-3,4-dihydro-2H-naphtho[2,1-e][1,3,4]oxadiazine-5,6-dione (22)

2,3-Epoxy-1,4-naphthoquinone (5 mmol) was added to a solution of **2** (5 mmol) in absolute ethanol. The reaction mixture was refluxed in water bath for 2 h. The precipitate that formed was filtered off. This solid was dissolved in distilled water then cooled to 0°c and dil. HCl was dropwise added to the solution until reached to PH 6. The crude product was filtered off, washed with water, dried, recrystallized from methanol **to** give buff crystals of 22 in 89 % yield, mp 185-186 °C.

IR (KBr, ν/cm^{-1}):3450 (2NH), 1687 (2C=O).^{1}H NMR (DMSO-d6) δ ppm 1.2-1.5 (m, 10H, H-aliph), 6.1(s, 1H, NH) and 7.7-8.01 (m, 1H, H-Ar). ^{13}C-NMR (DMSO-d6): δ ppm 16.6-77, 110.99, 125.3, 125.93, 130.56, 131.87, 134.47, 159.48, 181, 24 and184.73. MS (EI): (m/z, %), 296 (M^+, 1.5).

Biological activity

The following materials were used: Dulbecco's modified Eagles medium (DMEM) (Lonza, Belgium), a fetal bovine serum (FBS) (Lonza, Belgium), an antibiotic and antimycotic (penicillin-streptomycin) (Lonza, USA) sample, a Kreps ringer bicarbonate buffer (Sigma Aldrich, USA), a trypsin/EDTA 0.25 % (200 mg/L EDTA, 170,000 U trypsin/L) (Lonza, Belgium), a bovine serum albumin (BSA) (Hyclone, USA). , a phosphate buffer saline 1X (PBS) (Hyclone, USA), Trypan blue 0.4 % (Lonza, USA), an MTT assay kit (Lonza, USA), Ciprofloxacin (Sigma Aldrich, USA), Cisplatin (Hospira UK Ltd.).

MCF7 and normal cell lines were obtained from VACSERA, EGYPT. Candida Albicans Fungus was obtained from Regional Center of fungi, Al-Azhar University, Egypt. Gram +ve *Staphylococcus Aureus* and Gram –ve *E. Coli* were obtained from Department of Biotechnology, Faculty of Post Graduate Studies for Advanced Sciences, Beni-Suef University, Egypt.

Anticancer activity

The cells were cultured in DMEM medium at 37 ° C, 5 % CO_2 supplemented with 10 % fetal bovine serum (FBS), 100 U mL-1 penicillin and 100 µg mL^{-1} streptomycin. Cell viability was estimated using MTT assay Kit which based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye to violet formazan crystals by succinate dehydrogenase inside living cells mitochondria. The cells were seeded into 96-well plate with a concentration of 6000 cells/well and incubated for 24 h. The medium was discarded and various concentrations of the test compounds dissolved in DMEM were added to wells. After another incubation with the same conditions, the medium was discarded, and 100 µL MTT (2 mg mL^{-1}) was added and incubated for 3 h at 37 °C. The produced purple formazan crystals were dissolved in 50 µL of DMSO. The plate was then incubated for 15 min at 37 °C and the optical density was read at 570 nm with a reference wavelength 630 nm as using Stat fax Elisa plate reader (Stat fax, USA). Cisplatin (Hospira UK Ltd.) was used as positive control and DMSO was used as the solvent for compounds and its final concentration was less than 0.2%. IC50 and IS50 were calculated. All in vitro tests were performed in triplicates.

Antimicrobial and antifungal activity tests

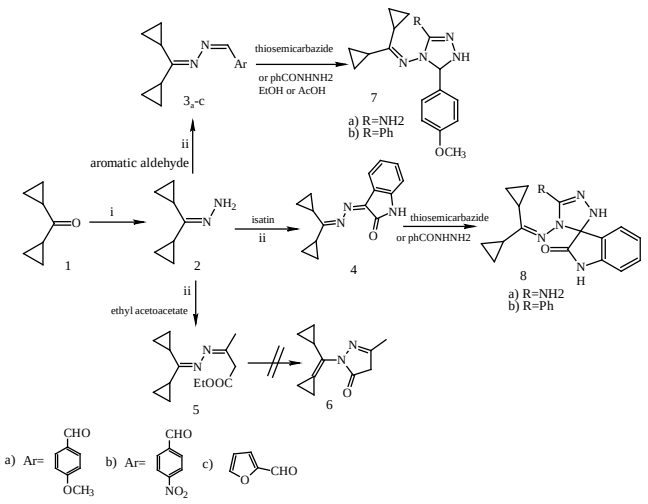
The studied compounds were diluted with DMSO to conc. 100 mg mL^{-1} then about 6mm diameter filter paper was soaked in 75 µl of diluted compounds. Ciprofloxacin (20 µg mL^{-1}) was used as positive control and DMSO was used as negative control. Nutrient agar plates were inoculated with Gram +ve *Staphylococcus Aureus*, Gram –ve *E. Coli* and *Candida Albicans* by sterile swabs and the tested compound containing discs were placed onto the nutrient agar surface by sterile forceps. Plates were incubated at 37°C/24 h. Inhibition zone was measured in mm. Tests were performed in triplicates.

Result and discussion

The target synthetic products **2–23** were prepared using synthetic procedures are summarized in Schemes 1-4. Condensation of dicyclopropyl ketone **1** with hydrazine hydrate in refluxing ethanol afforded the key hydrazone derivative (**2),** followed by condensation with appropriate aromatic aldehydes in refluxing ethanol formed the corresponding Schiff bases (**3-5)** in good yields. Similarly, the hydrazone **2** was condensed with isatin in the presence of a few drops of glacial acetic acid as a catalyst to produce the Schiff base **4** in a rather excellent yield. However, cyclization reaction of the hydrazine derivative **5** was unsuccessful.

The structures of the newly synthesized compounds **2-5** were confirmed on the basis of their spectral data (IR,^{1}H-NMR, ^{13}C-NMR and MS spectra). The infrared spectra of the formed hydrazones showed the absence of amino group and appearance of imino group at 1613-1659 cm^{-1}. In addition, ^{1}H-NMR spectrum of **3b** showed singlet signal at 3.79 ppm due to the methoxy group, while in the spectrum of compound **4** a singlet signal at 10.9 ppm for NH group was appeared. Also, the spectrum of compound **5** showed the presence of -$COOCH_2CH_3$ group, two protons appeared as a quartet at 4.0 ppm, three protons as a triplet at 1.22 ppm while, the three protons of the methyl group appeared as a singlet at 2.25 ppm.

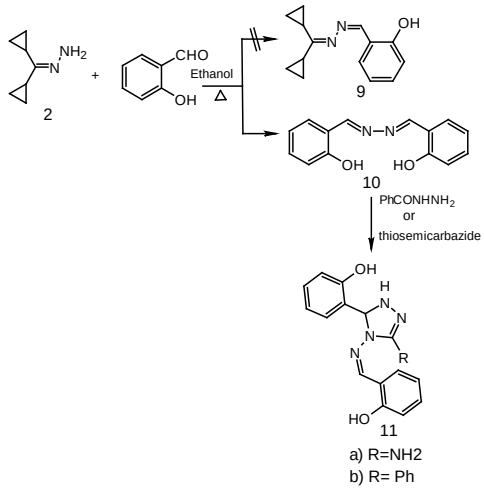
In view of the biological importance of triazoles,[23–27] our target was to synthesize new hydrazones attached to triazole moieties. Subsequent cycloaddition of imines **3a** and **4** with thiosemicarbazide or benzohydrazide in boiling ethanol in the presence of acetic acid furnished the triazole derivatives **7a, b** and **8a, b** respectively. Analytical and spectral data well characterized all the synthesized compounds.



Scheme 1. Synthetic route to obtain target hydrazone derivatives **3-5 (**(i) $NH_2NH_2.H_2O$, ethanol, reflux; (ii) ethanol, glacial CH_3COOH, reflux) and subsequent formation of triazole derivatives by reaction of the synthesized Schiff bases with thiosemicarbazide and benzohydrazide

The amino protons in the sepctra of compounds **7a** and **8a** were appeared in the range of 9.8-12.9 ppm. All the aromatic protons were found in the range of 6.7-8 ppm. IR spectrum for compound **8b** ascertained its structure by the appearance of the characteristic bands at 3438 and 1691 cm^{-1} for the amino and carbonyl groups. Its ^{1}H-NMR spectrum showed singlet signals at 12.4 and 11.0 ppm assigned to 2NH protons. The appearance of aromatic hydrogen signals was confirmed by the multiplet signals in the range of 6.8-7.6 ppm. The ^{13}C-NMR and mass spectra were in good agreement with the molecular formula. On the other hand, with an unexpected reaction of salicylaldehyde with hydrazone **2** it gave the light yellow crystals of salicylaldehyde azine **10** with 95 % yield which was synthesized previously.[28] The proposed mechanism accompanied by degradation of the hydrazone group and reformation of the starting dicyclopropyl ketone then condensation of the resulting salicylaldehyde hydrazone with another mole of salicylaldehyde. Comparison with the reported spectra and melting point data with the synthesized compound gave us absolute confidence to assign it as salicylaldehyde diazine **10**.

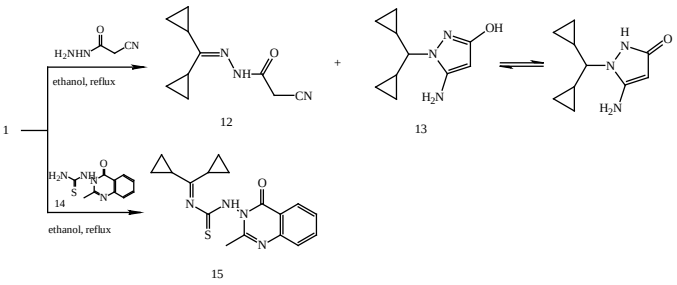
The spectral data of **10** were in good agreement with the expected structure of the compound. Its IR spectrum showed absorption bands at 3445 and 1622 cm^{-1} due to 2OH and 2C=N groups and absence of absorption band of NH_2 group. ^{1}H-NMR and ^{13}C-NMR revealed the absence of aliphatic protons and carbons of the cyclopropyl rings, respectively. Its mass spectrum exhibited a molecular ion peak at m\z 240 which consistent the molecular formula of the bis-azine **10.** We have attempted to prepare the Schiff base derivative **9** by the addition reaction of thiosemicarbazide and benzohydrazide with compound **2**. As the unexpected direction of the reaction led the formation of bis azine **10,** this compound was reacted with thiosemicarbazide and benzohydrazide to form triazoles **11a** and **11b**, respectively. Their structures were assigned by their spectral and analytical data. IR spectrum for **11a** showed the presence of NH_2 bands at 3173 and 3137 cm^{-1} and the mass and NMR spectra were confirmed the cyclization reaction with thiosemicarbazides. In the same way, the formation of **11b** could be confirmed by its spectral data.



Scheme 2. The reaction of hydrazine **2** with salicylaldehyde and subsequent with thiosemicarbazide and benzohydrazide

Compound **1** was reacted with cyanoacetic acid hydrazide in ethanol as a solvent given a mixture of cyanoacetohydrazone and pyrazole derivatives (**12** and **13)** which can exist in two tautomeric ketone-enol forms), respectively and these products were separated easily from ethanol by cooling. Treatment of dicyclopropyl ketone with thiourea derivative **(14)** in boiling ethanol gave the corresponding quinazoline derivative (**15)**.

The structures of the **12** and **13** were elucidated on the basis of their IR, ^{1}H-NMR, ^{13}C-NMR and MS spectra. For example, the IR spectrum of **12** revealed the presence of absorption band at 2264 cm^{-1} characteristic for cyano group. There were no found any absorption bands at the region of cyano group, but there was found absorption bands at 3166 due to the presence NH_2 group in the spectrum of compound **13**. NMR spectra also confirmed the structures with appearance of methylene signals at 3.9 ppm and NH_2 signal at 10.8 ppm. Further, the structure of compounds **12-15** was established on the basis of analytical and spectral data.

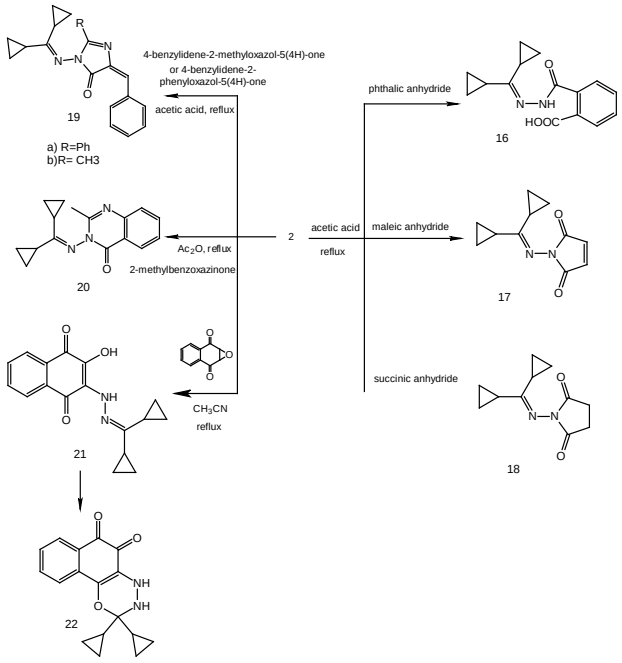


Scheme 3. reaction of dicyclopropyl ketone 1 with cyanoacetic acid hydrazide and thiourea derivative in absolute ethanol

Finally, the hydrazone derivative **2** was reacted with different anhydrides like phthalic, succinic and maleic anhydrides in refluxing acetic acid to give phtalamido (a ring opening of the anhydride was observed and attempts made to cyclize the synthesized product were failed), 1-succinimido and 1-pyrrole analogs **16-18**, respectively. The formation of compounds **16-18** could take place through the nucleophilic addition by the nitrogen nucleophile of the hydrazide on the carbonyl group of the anhydride leading to ring opening followed by cyclisation with the elimination of a water

molecule. The same methodology was extended to the preparation imidazolone derivatives **19a,b** by the reaction of **2** with appropriate oxazolones, respectively**.** Furthermore, the reaction of **2** with 2-methylbenzoxazinone gave quinazolinone **20** in 54 % yield. 2,2-Dicyclopropyl-3,4-dihydro-2H-naphtho[2,1-*e*][1,3,4]oxadiazine-5,6-dione (**22**) was prepared by condensation of (dicyclopropyl-methylene)hydrazone (**2**) with 2,3-epoxy-1,4-naphtho-quinone in boiling acetonitrile in excellent yield through the formation of intermediate **21** with subsequent cyclization.

Structural characterizations of the synthesized compounds **14-22** were performed by IR, ^{1}H and ^{13}C NMR and mass spectrometry. The IR spectra of compounds **17** showed the absence NH_2 absorption band and the presence of characteristic amide -C=O group stretching bands around 1739 cm^{-1}. IR of **16** showed a distinctive band at 1746 cm^{-1} due to the carbonyl of carboxylic group. Its ^{1}H-NMR spectrum showed the presence of aromatic protons and exhibits a singlet signal at 11.5 ppm due to proton of COOH group. Analysis of the ^{13}C-NMR spectra of the hydrazones **16-17** revealed the presence of a signal for the carbonyl group of amide groups at approximately 172 ppm. The mass spectra of compounds **16-17** revealed the presence of the molecular ion peaks. IR and NMR spectra confirmed the structures of **19a,b** and **20** by the disappearance of the NH_2 group signals besides appearance of the expected signals. The structure of the synthesized compound **22** was ascertained on the basis of its IR, mass and NMR spectral characteristics. The presence of NH and C=O functional groups was marked by the appearance of stretching bands at 3450 and 1678 cm^{-1} respectively. Its ^{1}H-NMR spectrum showed two singlet signals at 6.3 and 11.6 ppm characteristic for two NH protons and the carbonyl signals are appeared at 184.73 and 181.24 ppm in the ^{13}C-NMR spectrum.



Scheme 4. Reaction of hydrazone derivative 2 with anhydrides, oxazolone, benzoxazinone and epoxy-1,4-naphthoquinone derivatives

Antimicrobial activity

The newly synthesized compounds were evaluated for their antibacterial and antifungal activities against *Staphylococcus Aureus, Escherichia coli* and *Candida Albicans.* Ciprofloxacin was used as reference material for the comparison. The results for antimicrobial activity showed that compounds **10, 19a** and **20** exhibited higher bioactivity against *Staphylococcus* than the standard material and compounds **3b, 3c, 4, 11a, 11b, 12, 13, 15** and **7a** showed moderate activity and compounds **5, 16** and **8b** possessed excellent antibacterial activity against *E. Coli*. Most of the synthesized compounds showed high to good antifungal action against *C. albicans* growth in comparison with the reference material. Results showed that compounds **3b, 3c, 15** and **7a** were more active among all the test compounds followed by compound **9b** and **18**. The synthesized compounds **4, 12, 19b** and **20** did not show any antifungal activity at the studied concentrations against *C. albicans* growth.

Table 1: Antimicrobial activity of the synthesized compounds

Compounds	**Antimicrobial activity inhibition zone in mm**		
	Staphylococcus Aureus	***E. Coli***	***Candida albicans***
Ciprofloxacin	12	9	9
2	19	17	18
3a	15	21	15
3b	-	16	25
3c	16	12	22
4	18	-	-
5	-	25	15
7a	19	22	22
7b	10	18	12
8a	10	15	13
8b	-	24	18
10	24	15	18
11a	16	10	15
11b	17	22	20
12	16	22	-
13	15	19	9
15	17	22	15
16	-	24	21
18	10	-	12
19a	20	22	15
19b	-	-	-
20	21	15	-
22	11	18	10

Anticancer activity

The anticancer activity for the synthesized compounds was evaluated in vitro screening against breast cancer cell line (MCF-7) (Table 2). Cisplatin was used as the reference material. From the results of Table 2, it was found that compound **4** exhibited the most excellent anticancer activity for MCF-7 cell lines, with an IC_{50} value of 21.5 it proved to be much more active than the reference drug and compared to most of the tested compounds. This is may be due to the presence of indole moiety.

Furthermore, compounds **13, 18** and **19a** were found to be more active than the reference drug also with IC_{50} 22.4, 23.6 and 25.5, respectively. The cell killing potency of **13** is high and this may be due to the presence of two active free functional groups OH and NH_2 attached to pyrazole moiety. While the presence of imido and imidazolone skeletons and the presence of phenyl groups enhanced their cytotoxic activity of **18** and **19a** respectively. On the other hand, the other tested synthesized compounds were exhibited moderate to weak cytotoxic activity.

The selectivity index (SI) for our newly synthesized drugs was calculated by comparison the cytotoxic effect of drugs against cancer cell line with its cytotoxic effect against human lung fibroblast normal cell line MRC-**5** and the findings came with high satisfaction, where compounds **4, 13, 18, 19a** showed selectivity to cancer cell line higher than cisplatin selectivity, also compound **2** showed the same selectivity in comparison with cisplatin.

Moreover, compounds **3a, 3c, 7a, 7b, 8a, 11b, 12, 19b** showed a satisfactory selectivity index, especially **8a** that was closer to cisplatin selectivity index. These results proved that our newly synthesized hydrazone derivatives were selective and specific to hepatocellular carcinoma cell line HEPG2 more than normal human cell line.

Table 2. Cytotoxicity of cisplatin and the new compounds against MCF-7 breast cancer cells and normal cell line

MTT assay IC50 24h (µM)			***SI***
Compounds	**MCF7**	**MRC-5**	
Cisplatin	28.4	90	3.1
2	62.8	200	3.1
3a	50	120	2.4
3b	100	>200	-
3c	50.5	150	2.9
4	21.5	75	3.4
5	>100	150	-
7a	40.8	90	2.2
7b	51.7	120	2.3
8a	50	150	3
8b	84.2	100	1.1
10	90.6	150	1.6
11a	80.2	85.2	1.06
11b	45.2	100	2.2
12	55.8	150	2.6
13	22.4	80	3.5
15	48.6	>200	-
16	>100	95	-
17	100	150	1.5
18	23.6	100	3.2
19a	25.5	84.7	3.3
19b	75.4	175	2.3
20	50	75	1.5
22	>100	50	-

IC_{50}: drug concentration that inhibits cell growth by 50%. *SI* was calculated by dividing IC_{50} value of lung fibroblast normal cell line for each compound against IC_{50} of the cancer cell line**.**

Conclusion

Some new hydrazones based on (dicyclopropylmethylene)hydrazine **2** were synthesized and transformed into a series of heterocyclic products such as imidazolone, quinazolinone, triazole and indole derivatives. The antimicrobial and antitumor activities of the newly synthesized compounds were evaluated. Most of the compounds showed higher activity compared with Ciprofloxacin as positive controls and some of the compounds showed satisfactory activity against a breast cancer cell line (MCF7) compared with cisplatin as a positive control.

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Received: 15.11.2018.
Accepted: 30.12.2018.

SYNTHESIS AND CHARACTERIZATION OF NEW THIAZOLIDINONES AND 2-OXOPYRROLIDINES DERIVED FROM SCHIFF BASES

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Keywords: thiazolidinones; five-membered γ- lactams; imines.

New thiazolidinones and γ- lactams were prepared from mixtures of Schiff base (imine) and thioglycolic acid or phenylsuccinic anhydride, respectively, in moderate yields (52-71 %). The structures of these new thiazolidinones and γ-lactams were established on the basis of the IR, ^{1}H-NMR,^{13}C-NMR, ^{13}C-NMR DEPT and mass spectral data.

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Introduction

Thiazolidinones (Figure 1a) are classified as doubly unsaturated five-membered heterocyclic compounds contain one nitrogen, one sulfur and three carbon atoms including a carbonyl group. Thiazolidinones and their derivatives show a large variety of biological activities such as antibiotic, diuretic, tuberculostatic, organoleptic, antileukemic and antiparasitic.[1,2] As far as literature is concerned, only a few information is available about thiazolidinones and their bioactivity. The chemistry of thiazolidin-4-one ring system is considerable interest because it is the core structure in various pharmaceuticals.

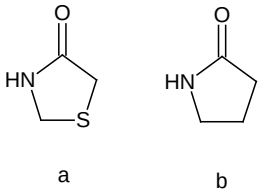


Figure 1. Structure of thiazolidinones and 2-oxopyrrolidines (γ-lactams).

Five-membered ring lactams, which are known as γ-lactams or 2-oxopyrrolidines (Figure 1b), are essential structural motifs in biologically active natural products and used in medicines and approved drugs.[3] γ -Lactams have attracted considerable attention in recent years because they are valuable building blocks in the structure of several biologically active molecules.[4] Substituted γ-lactams, in particular, have potential application in drug synthesis, but the development of the stereoselective synthesis of chiral γ-lactams remains a challenge.[5,6] Various γ-lactams are components of natural products,[7] and some biologically important lactams[8] are obtained from the reaction of imines with phenylsuccinic anhydride.

Experimental part

All solvents were distilled/dried prior to use, whenever this seemed necessary, by standard methods. All solvent extracts were dried over anhydrous sodium sulfate unless otherwise specified.

FT-IR spectra were recorded using a Shimadzu FT-IR spectrophotometer as KBr. The absorption bands of interest are reported and expressed in cm^{-1}.

^{1}H-NMR spectra were recorded using a Bruker Varian NMR spectrometer (500 MHz). The chemical shift values are expressed in δ(ppm), using tetramethylsilane (TMS) as internal standard and DMSO-d_6 as a solvent. ^{13}C-NMR spectra and ^{13}C-NMR DEPT spectra were recorded using a Bruker Varian spectrometer (75 MHz). The chemical shift values are expressed in δ(ppm), δ(ppm), using tetramethylsilane (TMS) as internal standard and $CDCl_3$ as a solvent.

Mass spectra were recorded using a 70 eV HPLC-LCQ Fleet/Thermo Scientific instrument with 5973 type mass selective detector.

General procedure for preparation of imines

In general, the imines (**2a-2d**) were prepared by reaction the corresponding amines with an aldehyde or a ketone in 40 mL of methanol and 4-6 drops of glacial acetic acid with refluxing the reaction mixtures for 1-5 h under stirring. The progress of the reaction is followed by TLC. After completion the reaction, the solvent was evaporated then the residue was recrystallized from a suitable solvent. The physical data of the prepared imines (**2a-2f**) are gathered in Table 1.

3-Bromo-2-(pyridin-2-yliminomethyl)phenol (2a)

This compound was prepared by reacting of 2-aminopyridine (0.01 mol, 1 g) with 5-bromo-2-hydroxybenzaldehyde (0.01 mol, 2.4 g). R_f=1.2. Yield = 79.6 %, m.p. = 138-139 ºC. IR (KBr disk): 1610 cm^{-1} (C=N).

3-Bromo-2-(pyridin-3-yliminomethyl)phenol (2b)

This compound was prepared by reacting of 3-aminopyridine (0.01 mol, 1 g) with 5-bromo-2-hydroxybenzaldehyde (0.01 mol, 2.4 g). R_f=0.4, yield was 81.6 %, m.p. = 125-126 ºC. IR (KBr disk): 1615 cm^{-1} (C=N).

4-(5-Aminonaphthalenylimino)pentan-2-one (2c)

This compound was prepared by reacting of 1,5-diamino naphthalene (0.006 mol, 1 g) with acetylacetone (0.006 mol, 0.63 g ,0.65ml). R_f=0.5., Yield = 52.6 %, m.p = 200 ºC. IR (KBr disk): 1612 cm^{-1} (C=N).

4-(4-Aminophenylimino)pentan-2-one (2d)

This compound is prepared by reacting of p-phenylenediamine (0.0099 mol, 1 g) with acetylacetone (0.0099 mol, 0.93 g, 0.95 ml). R_f=0.5, yield = 90.9 %, m.p = 94-95 ºC. IR (KBr disk): 1601 cm^{-1} (C=N).

4-(Pyridin-3-ylimino)pentan-2-one (2e)

This compound was prepared by reacting of 3-aminopyridine (0.01 mol, 1 g) with acetylacetone (0.01 mol, 1.06 g, 1.09 ml). R_f=2, yield = 93.5 %, b.p. = 129-132 ºC. IR (KBr disk): 1612 cm^{-1} (C=N).

3-(4-Chlorobenzylideneimino)pyridine (2f)

This compound was prepared by reacting of 3-aminopyridine (0.006 mol, 1 g) with p-chlorobenzaldehyde (0.006 mol, 2.54 g). R_f=2, yield = 97 %, m.p = 73-74 ºC. IR (KBr disk): 1623 cm^{-1} (C=N).

General procedure for preparation of thiazolidinones (3)

A mixture of compound **2a-2d** and thioglycolic acid in chloroform (15) ml was allowed to react in a Teflon beaker in a microwave oven at 100 W for 6-12 minutes. Progress of the reaction is checked by TLC using hexane-ethyl acetate as eluent. After the completion of reaction, chloroform was removed by distillation to give a solid residue. The solids were washed successively with 1 N HCl (20 mL), water (2×20 mL), 5% $NaHCO_3$ (20 mL) and brine (20 mL). The organic layer was dried with Na_2SO_4 The solvent was removed by evaporation. The following thiazolidinones were prepared:

2-(2-Bromo-6-hydroxyphenyl)-3-(pyridin-2-yl)-thiazolidin-4-one (3a)

This compound was prepared by reacting **2a** (0.003 mol, 1 g) and (0.003 mol, 0.33 g, 0.25 mL) of thioglycolic acid. R_f=0.7, yield = 63 %, m. p. = 88-89 ºC. IR (KBr disk): 1673 cm^{-1} (–N–C=O). ^{1}H-NMR (500MHz, DMSO-d_6, δ, ppm) 3.81(d, 1H), 4.19(d, 1H), 5.68(s, 1H), 6.15-7.51 (m, 3H), 6.09-8.12 (m, 4H) and 10.21 (s, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$, δ, ppm) 38.65, 48.02, 122.03-151.02 and 177.86

2-(2-Bromo-6-hydroxyphenyl)-3-(pyridin-3-yl)thiazolidin-4-one (3b)

This compound was prepared by reacting **2b** (0.003 mol, 1 g) and (0.003 mol, 0.33 g, 0.25 mL) of thioglycolic acid. R_f=1.2, yield = 71 %, m. p. = 98-99 ºC. IR (KBr disk): 1672 cm^{-1} (–N–C=O). 1H-NMR (500 MHz, DMSO-d_6, δ, ppm) 3.91(d, 1H), 4.31(d, 1H), 5.61(s, 1H), 6.22-7.51 (m, 3H), 6.01-8.12 (m, 4H) and 10.26 (s, 1H). ^{13}C NMR (75 MHz. $CDCl_3$, δ, ppm) 42.96, 52.12, 128.13-133.30, 135.95-151.34 and 180.52.

3-(5-Aminonaphthalen-1-yl)-2-methyl-2-(2-oxopropyl)-thiazolidin-4-one (3c)

This compound was prepared by reacting **2c** (0.001 mol, 0.41 g) and (0.001 mol, 0.157 g, 0.12 mL) of thioglycolic acid. R_f=0.6, Yield = 69 %, m. p. = 139-140 ºC. IR (KBr disk): 1676 cm^{-1} (–N–C=O). 1H NMR (500 MHz, DMSO-d_6, δ,ppm) 3.88 (d, 1H), 4.07 (d, 1H), 1.90 (s, 3H), 2.79 (s, 3H), 4.29 (s, 2H), 4.60 (s, 2H) and 7.30-8.02 (m, 6H). ^{13}C-NMR (75 MHz, $CDCl_3$, δ, ppm) 40.80, 61.85, 18.28,26.78, 36.95, 124.44-156.76, 179.17 and 177.86.

3-(4-Aminophenyl)-2-methyl-2-(2-oxopropyl)-thiazolidin-4-one (3d)

This compound was prepared by reacting **2d** (0.005 mol, 1 g) and (0.005 mol, 0.48 g, 0.36 mL) of thioglycolic acid. R_f=1.2, Yield =57 %, m. p. = 86-87 ºC. IR (KBr disk): 1681 cm^{-1} (–N–C=O). ^{1}H NMR (500 MHz, DMSO-d_6, δ,ppm) 3.98 (d, 1H), 4.25 (d, 1H), 1.89 (s, 3H), 2.85 (s, 3H), 4.65 (s, 2H), 4.75 (s, 2H) and 7.55-7.73 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm) 18.38, 26.75, 35.92, 40.82, 61.55, 124.66-152.78, 178.15 and 201.15.

General procedure of γ-lactams (4)

In general the γ-lactam were prepared by reaction the mixture of imines 2a, 2b, 2e and 2f) with phenylsuccinic anhydride in 20 mL of chloroform, then the mixture was refluxed for 1-12 h with stirring. The progress of the reaction was followed by TLC. After completion the reaction, the solvent was evaporated and the residue was recrystallized from ethanol. The following γ-lactams were prepared:

2-(2-Bromo-6-hydroxyphenyl)-5-oxo-3-phenyl-1-(pyridin-2-yl)pyrrolidine-3-carboxylic acid (4a)

This compound was prepared by reacting **2a** (0.003 mol, 1 g) and (0.003 mol, 0.64 g) of phenylsuccinic anhydride. R_f=0.7, yield = 55 %, m. p. = 121-122 ºC. IR (KBr disk): 1638 cm^{-1} (–N–C=O), 1727 cm^{-1} (HO–C=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm) 3.28 (d, 1H), 3.52 (d, 1H), 4.12 (s, 1H), 6.15-6.70 (m, 5H), 7.63-8.35 (m, 7H), 10.31 (s, 1H) and 11.42 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm) 42.88, 53.02, 59.79, 121.23-158.52, 125.95-135.95, 127.89-138.11, 172.13 and 185.88.

2-(2-Bromo-6-hydroxyphenyl)-5-oxo-3-phenyl-1-(pyridin-3-yl)pyrrolidine-3-carboxylic acid (4b)

This compound was prepared by reacting **2b** (0.003 mol, 1 g) and (0.003 mol, 0.64 g) of phenylsuccinic anhydride. R_f=0.6, yield = 61 %, m. p. = 113-114 °C. IR (KBr disk): 1655 cm^{-1} (–N–C=O), 1719 cm^{-1} (HO–C=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm) 3.51 (d, 1H), 3.81 (d, 1H), 4.55 (s, 1H), 6.08-6.54 (m, 5H), 7.18-8.02 (m, 7H), 9.98 (s, 1H) and 11.40 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm) 41.56, 51.07, 57.96, 120.53-152.74, 124.45-133.90, 171.25 and 178.25.

2-(2-Bromo-6-hydroxy-phenyl)-5-oxo-3-phenyl-1-(pyridin-3-yl)pyrrolidine-3-carboxylic acid (4e)

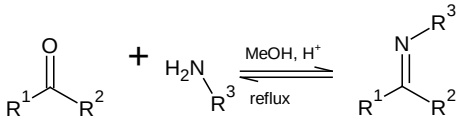
This compound was prepared by reacting **2e** (0.005 mol, 1 g) and (0.005 mol, 1 g) of phenylsuccinic anhydride. R_f=0.5, yield = 68.4 %, m. p. = 168-169 °C. IR (KBr disk): 1602 cm^{-1} (–N–C=O), 1697 cm^{-1} (HO–C=O). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm) 3.25 (d, 1H), 3.56 (d, 1H), 1.91 (s, 3H), 2.28 (s, 3H), 3.98 (s, 2H), 6.21-8.08 (m, 9H), and 11.07 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm) 16.02, 27.23, 37.84, 41.12, 52.11, 59.23, 121.66-157.17, 125.33-139.17, 168.94, 180.73 and 206.85.

2-(4-Chlorophenyl)-5-oxo-3-phenyl-1-(pyridin-3 -yl)-pyrrolidine-3-carboxylic acid (4f)

This compound was prepared by reacting **2f** (0.0046 mol, 1 g) and (0.0046 mol, 0.8 g) of phenylsuccinic anhydride. R_f=0.1, yield =55 %, m. p. = 159-160 °C. IR (KBr disk): 1602 cm^{-1} (–N–C=O), 1695 cm^{-1} (HO–C=O). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm) 3.32 (d, 1H), 3.56 (d, 1H), 4.53 (s, 1H), 6.21-6.64 (m, 5H), 7.30-8.20 (m, 7H), and 11.40 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm) 40.13, 51.28, 58.54, 121.67-151.66 124.56-138.54, 169.18 and 183.05.

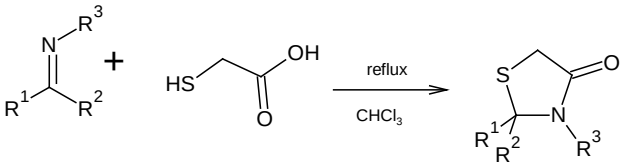
Results and discussion

The Schiff bases are formed by the condensation of primary amines and an aldehyde or ketone.



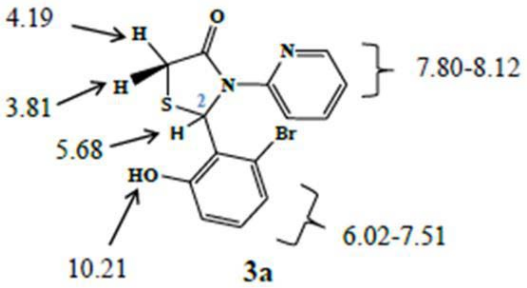
A simple synthetic way to prepare the biologically active thiazolidinones[9,10] is based on the reaction of imines with thioglycolic acid:

The IR spectra of imines **2a-2f** made is characterized by four principal band groups correspond to the stretching vibrations of the aromatic C-H bonds, aliphatic C-H bonds, azomethine bonds (C=N), and aromatic C=C bonds of the and substituted aromatic ring, which occur within the ranges of 3224-3047, 3007-2777, 1638-1610, and 1586-1475 cm^{-1}, respectively**.**

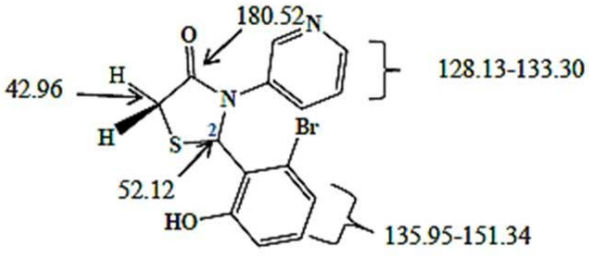


Six band groups can characterize the IR spectra of thiazolidinones 3a-3d correspond to the stretching vibration of the aromatic C-H, aliphatic C-H, carbonyl amide group (N-C=O), aromatic C=C bonds, C-N bonds, and bending vibration of C-S bond, which occurs within the ranges of 3369-3050, 2946-2800, 1720-1672, 1631-1546, 1282-1271 and 778-626 cm^{-1}, respectively.

The ^{1}H-NMR spectrum of 2-(2-bromo-6-hydroxyphenyl)-3-(pyridin-2-yl)thiazolidin-4-one (**3a**) (see Electronic Supporting Information) is evaluated as an example. The signal at chemical shift δ 2.50-2.51 ppm belongs to the DMSO-d_6 solvent. There are two doublet signals at chemical shift δ 3.81 ppm (J=3 Hz) and δ 4.19 ppm (J=3Hz) for methylene protons of thiazolidin-4-one ring (Fig.1). The chiral carbon atom (No.2) gives a singlet signal at δ 5.68 ppm (racemic mixture (R, S) configuration) and there is a multiple signal system for three protons of the phenol ring at δ 6.02-7.51. A multiplet signal for four protons of pyridine ring at δ 7.80-8.12 ppm also appears.

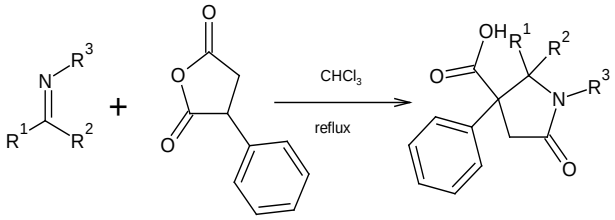


The ^{13}C NMR spectrum of 2-(2-bromo-6-hydroxyphenyl)-3-(pyridin-3-yl)thiazolidin-4-one (**3a)** as example **(**see Electronic Supplementary Information**)** shows a signal at δ 76.34-76.96 ppm for the $CDCl_3$ solvent. There are two singlets at δ 42.96 ppm and 52.12 for the methylene group carbon and No.2 carbon of the thiazolidin-4-one ring. A multiplet signal between δ 128.13-151.34 ppm for aromatic carbon atoms (pyridine + phenol) ring and a singlet signal at δ 180.52 ppm for the carbon of the amide carbonyl group are also identified.



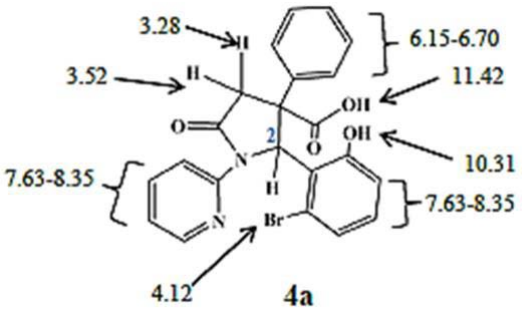
The ^{13}C-NMR DEPT spectrum of **3a** shows a signal at δ 48 (-) ppm for (CH_2), and a signal at δ 38(+) ppm for CH carbon of thiazolidin-4-one ring. There are signals belong to the aromatic region in the range (125-135) (+) ppm and there are signals at δ 122-148 (+) and (177) (+) ppm for the pyridine ring and the amide carbonyl.

γ-Lactams also represent important substructures for the synthesis of biologically relevant compounds in drug discovery[11] and natural products.[12,13] The prevalence of these structures[14,15] resulted in the development of several efficient methods[16] and preparation of diverse libraries of small molecules for biological evaluation.[17,18] Based on these earlier studies, a practical way, the cyclization of imines with phenylsuccinic anhydride in chloroform was followed:



Bands characterize the IR spectra of γ-lactams **4a**, **4b**, **4e** and **4f** belong to the stretching vibrations of the carboxylic OH, aromatic C-H, aliphatic C-H, carboxylic carbonyl group, carbonyl amide group, aromatic C=C and substituted aromatic ring in the ranges of 3134-3026, 3064-2742, 1727-1695, 1651-1586, 1602-1536 and 939-809 cm^{-1}, respectively.

The ^{1}H-NMR spectrum of 2-(2-bromo-6-hydroxyphenyl)-5-oxo-3-phenyl-1-(pyridin-2-yl)pyrrolidine-3-carboxylic acid (**4a)** (see Electronic Supplementary Information) showed signal at δ 2.57-2.58 ppm belongs to DMSO-d_6 solvent. Two doublet signals are appeared at δ 3.28 ppm and 3.52 ppm, (J=4 Hz) for methylene protons of pyrrolidine ring, a singlet at δ 4.12 ppm for the proton of the chiral carbon (No 2) atom (racemic), and two multiplet signals for the five protons of benzene ring at δ 6.15-6.70 and δ 7.63-8.35 for seven aromatic protons of phenol and pyridine rings, respectively.



There are two singlet signals at δ 10.31 ppm and 11.42 ppm of phenol and carboxyl hydroxy groups, respectively.

The mass spectrum of **4a** showed the molecular ion peak in 453,455 m/z and important fragmentation peaks at m/z=452, 454 m/z=424, 426, m/z, 399, 401, m/z= 382, 384, m/z=354, 356 m/z (these fragments contains two bromine isotopes), and fragments without bromine isotopes at m/z, 223, 181 103 78, 77 and 65.

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Received: 11.12.2018.
Accepted: 01.01.2019.

ANTIFUNGAL AND ANTI-TERMITE ACTIVITIES, TOTAL PHENOLIC CONTENT OF *PROSOPIS FARCTA* EXTRACTS; ATTEMPTS TO DEVELOP WEED BIOCONTROL METHOD AGAINST IT

Abdullatif Azab[a,b]

Keywords: Prosopis farcta, antifungal, anti-termite, total phenolic content, flavonoids, biocontrol.

Prosopis farcta is a widespread weed in the Near East and its an invasive plant of southwestern parts of the USA. Despite being sufficiently studied in the past, some of its activities were not published. In this research, we studied the antifungal and anti-termite activities of four extracts of the plant aerial parts: aqueous, ethanolic, ethyl acetate and hexane. In addition, since the published reports of total phenolic content (TPC) are not consistent, we tested this as well. We also tested the potential of the aqueous extract of *Carya illinoinensis* as possible weed biocontrol against *P. farcta*. The n-Hexane extract had the highest antifungal and anti-termite activities. TPC was found around 13.9 mg of gallic acid equivalent for 1 g of dry ethanolic extract (highest). The attempts to use an aqueous extract of *C. illinoinensis* for weed biocontrol of *P. farcta* achieved very limited success

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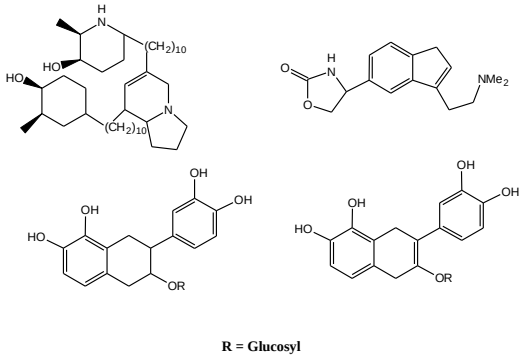
Introduction

Prosopis farcta is one of the most widespread plants over the southwestern regions of Asia, and it is n invasive plants in eastern USA.[1] It belongs to the *Fabaceae* family and the genus of Prosopis that includes 44 species.[2] Archeological findings indicate that ancient peoples of the eastern Mediterranean basin used this plant, mainly for food.[3,4] Most cultures of the Middle East have used *P. farcta* in their traditional medicines. One of the notable known uses is for the treatment of diabetes.[5] In Pakistan, it is reported that traditional societies use the plant for many purposes such as medicinal (humans and animals), animal food and fencing.[6] In the folk medicine of Jordan, *P. farcta* is used as antispasmodic and analgesic.[7] But according to published data, it is evident that Iranian traditional medicine has used this plant more than others. The uses included: blood thinner, antidiabetic, sterilizer (hands), rashes treatment, anti-atherosclerosis and menstruation pain.[8-13]

P. farcta was mentioned in several review articles, but all of them are related to the Prosopis genus in general and none of them scans the literature known about this species in particular, despite the extensive knowledge about it. Prabha et al. reviewed phenolics of *Prosopis* and their potential pharmacological uses.[14] In addition to phenolics, they indicated some other interesting compounds such as alkaloids present in *P. juliflora*, see Figure 1.



R = Glucosyl

Figure 1: Pharmacologically active compounds found in P. juliflora (Ref. [14])

Persia et al. published an article about the toxicity of *Prosopis* species, but they do not indicate the source of toxicity, i.e., the toxic compounds.[15] Finally, a group of researchers from the USA published a review article of controlling Prosopis species, which are invasive plants in the New World. The interest focuses mainly of Prosopis species that grow as trees, and less of P. farcta, which is more of a low, shrubby plant type.[16]

Reviewing of chemical composition and properties

The complete, systematic chemical composition of P. farcta was never published. All published studies so far reported only partial compositions, where they mainly included groups of compounds (phenolics, fatty acid …etc.) or characterization of certain natural products, mostly previously known, such as phenolics and flavonoids.

In Table 1 we summarized the findings of these published studies.

Table 1. Reported findings of chemical composition studies of *P. farcta*

Methods and Findings
The dry powder prepared from the whole plant (excluding seeds) was extracted with various solvents and analyzed by HPLC or GCMS (for volatiles). Most isolated compounds were phenolics and there were slight differences between two locations. See Figure 2. [17,18]
Dry powder of fruits was extracted with water and ethanol. Analysis method is not indicated but compounds groups that were found are mainly phenolics. [19]
A Method was developed to increase the yield of anthocyanins in callus cultures. [20]
Methanolic extract of aerial parts was analyzed for major compounds families. Analysis methods are not indicated. [21]
Fatty acids composition of seeds oil was analyzed after preparing methyl esters. Linoleic, oleic and palmitic acids constitute more than 90 percent of the fatty acids. [22]
Quercetin content of the plant fruits was measured from several locations in Iran, after extraction with acidic hydromethanol. See Figure 2. [23]
Oil extracted from seeds was analyzed and found containing a high concentration of protein (18%), unsaturated fatty acids (UFA) and low total phenolic content (1.7 mgGAE g^{-1}). [24]
Aqueous extract of dry aerial parts was prepared and total phenolic content was measured and found 17.3 mgGAE g^{-1}. [25]
HPLC analysis of acetone and methanol (successively) extract of different parts of the plant, harvested from various locations. Many compounds were identified, none is new. [26]
Comprehensive analysis for compound groups and minerals was performed after various extractions of plant parts. [27]
Aerial parts of the plant were extracted with: methanol, then with, *n*-hexane, methylene chloride, ethyl acetate and *n*-butanol. All extracts were analyzed mainly using GCMS. Many compounds were identified, none new. [28]

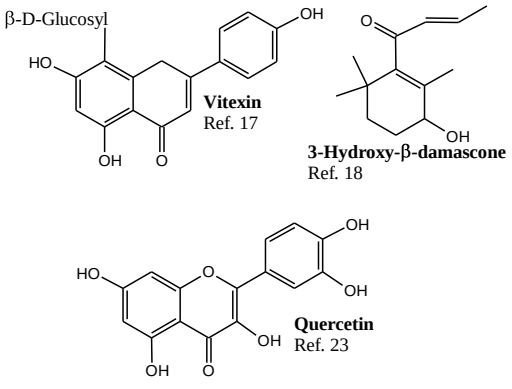


Figure 2: Selected compounds found in P. farcta

Table 2. Medicinal activities of *P. farcta* and their related properties

Tested Activities, Methods, Findings
Antibacterial The dry powder of fruits was extracted with H_2O and EtOH, and extracts were tested against antibiotic-resistant bacteria. Extracts had moderate activity.[19] MeOH extract of aerial parts was prepared and tested against antibiotics resistant bacteria and was found active.[21] Aerial parts of the plant were extracted with various solvents and tested against different bacteria. Each extract was more active than others against specific bacteria.[28] MeOH and *n*-hexane extracts of aerial parts were prepared and tested against the major pathogenic bacteria of fish, including *A. hydrophila*, *Y. ruckeri* and *S. iniae*. MeOH extract was more active.[29] Aq. ethanolic and MeOH pods extracts were tested against *S. paucimobilis*. EtOH and MeOH were more active than aq. extract.[30] EtOH extracts showed high antimicrobial activity.[31]
Anticancer Among extracts of aerial parts that were prepared with six different solvents, the EtOAc extract had the highest activity.[28] All plant aq. EtOH (80 %, v/v) extract was active against HT-29 cancer cell lines.[32]
Antidiabetic Aqueous extract of aerial parts was found active α-Glucosidase and α-Amylase inhibitor.[25] STZ-induced diabetes in male rats was treated with ethanol/water (70-80%) or methanolic various plant parts extract, or directly fed with solid extract. Clear activity was observed.[33-39,48] Diabetes was induced in cell lines with various agents and treated with plant infusion and *n*-hexane and acetone extracts. All plants products had glucose lowering effect.[40]
Antihyperlipidemic Blue-neck male ostriches were fed with seeds for 30 days and various bioactive materials were monitored in their blood. HDL cholesterol, total protein, and globulins levels increased, whereas LDL cholesterol, inorganic phosphorus, and γ-GT activity decreased.[41] Aqueous root extract reduced lipids in the blood of high cholesterol diet rabbits and had aorta protective effect.[42,43] Aqueous root extracts were active lipids lowering in rabbits livers.[44]
Antioxidant Methanolic extract of seeds was prepared and tested using three methods: TAC, DPPH, ABTS. Highly active.[24] Aqueous extract of aerial parts was tested with FRAP and ORAC methods and found moderately active.[25] Various extracts of aerial parts (including ultra-sonic assisted) were prepared and tested for antioxidant activity (DPPH): moderate.[27] *n*-Butanol extract had the highest antioxidant activity (ABTS), among six extracts that were prepared using different solvents.[28] Ethanolic extract of fruits was tested for antioxidant activity (DPPH, FRAP, ABTS) and found highly active. Total phenolic contents wad determined (62 mg GAE g^{-1}) and 27 compounds were identified. None new.[31] 80% EtOH/H_2O leaves extract had clear antioxidant (lipids) effect on STZ-induced diabetes in rats.[38] 70% Aq. EtOH fruits extract had explicit activity (FRAP)[39]
Cardioprotective Aqueous root extract had aorta protective effect.[43,45] Aqueous root extract had blood pressure lowering effect.[46]

Fertility
70% Ethanol/water fruits extract improved fertility of diabetic rats[39]

Hepatoprotective
Hydro-alcoholic extract (plant parts unknown) reduced level of malondialdehyde in liver.[38] Root aqueous extract had significantly decreased rabbits liver injuries.[44] 80% Ethanol/water extract had a protective effect against acetaminophen-induced hepatotoxicity in rats.[47] Hydroalcoholic pod extract had malondialdehyde level (in the liver) lowering in STZ-diabetic rats.[48] The methanol extract of aerial parts was active against CCl_4-induced liver toxicity in rats.[49]

Wound healing
Fruit powder and its aqueous extract found effective in healing wounds in STZ-induced diabetic rats.[50,51] Wounds made in healthy rats healed faster after treatment with a mixture of *P. farcta* and Ghee (butter, Persian).[52]

Energy production
Biomass was produced from the plant, using sodium tetraborate as most efficient catalyst.[53]

Nanoparticles synthesis
Silver nanoparticles (AgNP's) were produced from $AgNO_{3(aq)}$, using *P. farcta* aqueous extract as a reductant. AgNP's were tested for antibacterial and/or antioxidant activity.[54,55] Gold nanoparticles (AuNP's) were produced by the reduction of $HAuCl_4$ with aqueous leaves extract, and tested for anticancer activity.[56]

Reviewing medicinal activities and related properties

Most of the medicinal activities of *P. farcta* were studied and published. In addition to the classical activities such as antioxidant, antibacterial and antidiabetic, many other properties were also published, including preparation of nanoparticles. In Table 2 we summarized these published reports, mostly sorted by alphabetical order for the convenience of interested readers.

Prosopis farcta as a weed and weed control methods

The genus of *Prosopis* is described by some authors (USA) as "one of the world's worst woody invasive plant taxa".2 In Iran, *P. farcta* is considered as one of the worst weeds that grow wildly, mostly in the best and most fertile agricultural lands, and by this, it prevents the growth of many crops.[57,58]

Weed control is a worldwide issue, and 2016, the global spendings of weed control exceeded $B 40, not including manual weed unrooting.[59] All known methods used to control Prosopis species are based on chemical herbicides.[60] In Jordan, where P. farcta is a severe national problem, many synthetic herbicides were used separately and in combinations, but none of these methods proved successful.[61]

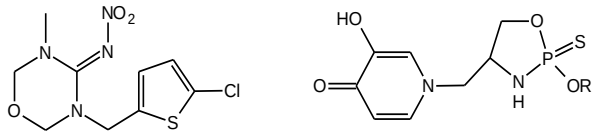
In addition to short term health concerns of chemical weed control (toxicity), it has significant adverse long term effects.[62] To avoid them, many efforts are being invested in the research of biocontrol of weeds, with a clear preference of environment-friendly methods. In Iran, studies were conducted to control *P. farcta* by using it as food for Nephopterygia austeritella (moth), with very limited success.[63] More successful were the attempts using another moth species, Stator limbatus, but also with limited success and method complexities.[64]

To the best of our knowledge, no plants material was used for biocontrol of *P. farcta* as published in the case of some other weeds.[65]

Antifungal and anti-termite activities of plant materials

In our previous publications,[66,67] we reported the antifungal activities of four plants. We also compared the use of synthetic antifungal agents with plant materials (extracts and pure natural products) that have the same property, and we showed the advantages of plant materials. Special attention was paid to reported antifungal activity against Rhizopus stolonifera (black mold), the same fungus that we tested the extracts of *P. farcta* against it.

Termites are social insects that belong to the Isoptera infraorder.[68] They cause major damages to forests,[69] buildings,[70] and many other wood-including facilities. In Israel, the most common species of termites is *Kalotermes sinaicus*.[71] Many synthetic chemicals were prepared and proved successful anti-termite agents.[72,73] Some of these compounds are shown in Figure 3.



Thiamethoxam **Mimosinol Derivatives**
R = Me, Et, *n*-Pr

Figure 3. Structures of some anti-termite synthetic compounds[72,73]

But as for most synthetic insecticides, these chemicals have many adverse side effects, such as toxicity to living organisms and damages to the environment. These effects lead to the development of plant-derived insecticides.[74] Many studies were published in recent years about this subject, and the research of F. Abdullah her colleagues from Malaysia is one of the most important.[75]

Experimental Section

Gallic acid was purchased from Merck Co. (Germany). All other chemicals were purchased locally in at least analytical grade.

Prosopis farcta (aerial parts) were harvested from the wild near our laboratory in Kfar-Qari (northern Israel). The green material was washed with distilled water and air dried for 4 weeks. The dry matter was ground into a fine powder and stored at -12 °C in sealed containers.

500 g of plant powder was stirred in 1000 mL of solvent (water, ethanol, ethyl acetate, n-hexane) for 24 h at 50 °C (n-hexane, 30 °C). Suspensions were allowed to cool to room temperature and filtered (Munktell quant. Grade 393) to obtain clear solutions. These were evaporated to dryness with rotary evaporator: aqueous extracts at 60 °C, ethanol, ethyl acetate and n-hexane extracts at 50 °C. All four extracts were solids, and they were stored in screw-capped vials at -12 °C. Extraction yields are shown in Table 3.

Antifungal activity tests

The antifungal assay was performed according to the method we reported in our previous publication, with no modifications.66 Rhizopus stolonifer was grown on whole wheat bread and extracted with water. The center of each Petri dish was inoculated with 5 mm diameter disc of fungal mycelium, taken from pure culture (7 days old). Then, all inoculated dishes were incubated at 25 °C for 6 days and the radial mycelial growth was measured. The antifungal activity of each extract was calculated in terms of the inhibition percentage of mycelia growth by using the following formula:

$$\% \text{ Inhibition} = [(d_c - d_t)/d_c] \times 100$$

where

d_c is the average increase in mycelia growth in control

d_t is the average increase in mycelia growth in treated samples with extracts

In all experiments, the control was the extraction solvent and we performed the antifungal tests using two concentrations for each extract: 10 % and 20 % (w/w). See Table 4 and Figure 4.

Total phenolic content test

The 2 L oxidative solution (1000 ml of 0.016 M sulfuric acid solution and 1000 ml of 0.004 M solution of KMnO4) was prepared according to the new method that we reported in our previous publication,67 with no changes. The final oxidative solution was prepared by combining the acid (0.016 M) and permanganate solution (0.004 M), which was stored in 4 °C in a sealed flask. Also, we used the calibration curve of gallic acid titration that we reported in the same publication. The titration of plant extracts was done according to the same method, with no changes. In a 100 ml Erlenmeyer flask that contained a magnetic stir bar, 100 mg of dry plant extract were suspended with 10 ml of distilled water and stirred for 5 minutes. The solution/suspension was titrated with the oxidative solution, with pH monitoring. Titration speed was 2 ml min^{-1}, with continuous gentle stirring. Results are shown in Table 5 and Figure 5.

The anti-termite activity of extracts

Anti-termite activity was performed according to the method that was reported by O. K. Ndukwe and his colleagues, with slight modifications.[76] Termites, *Kalotermes sinaicus*, were collected from infested tree trunk found in a nearby forest, put in glass trays and were immediately put in the Petri pre-prepared dishes.

Strips of filter paper (1x1 cm, Munktell quant. Grade 393) were saturated with 10% extract solution (w/v), each extract in its original extracting solvent. Then, paper strips were allowed to dry for 5 h. In the center of a 10 cm diameter petri dish, an extract loaded filter paper was placed with 10 termites, of unknown sex and age. Controls of this experiment were dry strips of filter paper loaded with solvents.

Termites were kept in these dishes for 4 weeks and were observed 3 times every day (07:00, 12:00 and 17:00). Mortality rate by the end of the 4 weeks was calculated as:

$$\%\text{Dead termites} = (\text{number of dead termites}) \times 10$$

Results are shown in Table 6 and Figure 6.

Attempts to develop biocontrol against P. farcta

500 g of fresh green peels of Carya illinoinensis were crushed (blender) to a homogeneous and soaked in 1 L of distilled water at 35 °C, for 24 h. Then the suspension was filtered and the filtrate was stored in a sealed bottle at 4 °C.

20 plants of *P. farcta*, most of the same height, were unrooted and planted in 20 identical flowerpots that each contained 2 kg of the same soil, that was brought from the same field (40 kg), and mixed before distribution into the pots. Each plant was fertilized with 5 g of potassium nitrate and irrigated with 500 ml of water. 10 of the plants were irrigated every 2 days with 20 ml of water each (control) and the other 10 were irrigated with 20 ml of *C. illinoinensis* extract every 2 days. All plants were kept in a laboratory hood under the same conditions of air flow, light and temperature. After 10 days, we tested the viability of the plants in two ways: if they are alive or dead, and if they are alive, are they partially dry or not. See Table 7 and Figures 7.

Results

Statistical analysis

Except for extractions (Table 3), that each was done in a single experiment, all data presented below, are average values of three experiments that we performed for each test.

Antifungal activity tests

Antifungal activity was measured as the inhibition percentage of mycelia growth of Rhizopus stolonifer. Two concentrations of extracts were used, 10% and 20% (w/w) in the extraction solvent and the results are shown in Table 4 and Figure 4.

Total phenolic content

Total phenolic content was determined by the method reported by us.[67]. Results are shown in Table 5 and Figure 5.

Table 3. Yields of extractions of *P. farcta* with four different solvents

Solvent	Water		Ethanol		Ethyl acetate		*n*-Hexane	
Yield	mass	%[a]	mass	%	mass	%	mass	%
	32.8	6.56	36.6	7.32	20.1	4.02	2.9[b]	0.58

Extraction yields: a - for each extraction, 500 g of dry plant powder (aerial parts) were extracted; b - since this yield is very low, we repeated this extraction three times and this is an average value.

Table 4. The antifungal activity of *P. farcta* extracts against *R. stolonifera*

Solvent	Water		Ethanol		Ethyl acetate		*n*-Hexane	
Extract Concentration (%, w/w)	10	20	10	20	10	20	10	20
Inhibition (%)[a]	18.2	22.1	26.3	27.9	26.3	27.8	32.5	36.8

a. Extraction solvent in each experiment was used as a control and resulted in 0 % inhibition.

Table 5. Total phenolic content of (TPC) extracts of *P. farcta*

Extract	Water	Ethanol	Ethyl acetate	*n*-Hexane
TPC[a]	12.4	13.9	10.9	5.1

a. mg of gallic acid equivalent in 1 g of dry extract

Table 6. The mortality rate of termites (*K. sinaicus*) as a result of feeding extracts loaded paper

Time (d)	----	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DR (%) WE	**TG**	0	0	0	0	10	10	10	20	20	20	30	30	30	30
	CG	0	0	0	0	0	0	10	10	10	10	10	10	10	20
DR (%) EE	**TG**	0	0	10	10	20	20	20	20	30	30	40	40	40	40
	CG	0	0	0	0	0	0	10	20	20	20	20	30	30	30
DR (%) EAE	**TG**	0	0	10	10	20	20	30	30	40	40	40	40	40	60
	CG	0	0	10	10	10	10	10	10	10	20	20	30	30	30
DR (%) HE	**TG**	0	10	10	20	40	50	70	80	90	100	100	100	100	100
	CG	0	0	0	0	10	10	20	20	20	20	20	20	30	30

DR, Death rate. WE, water extract; EE, ethanol extract; EAE, ethyl acetate extract, HE, n-hexane extract, TG, test group; CG, the control group

Table 7. *P. farcta* with dry branches after irrigation with aqueous extract *C. illinoinensis*

Number of Irrigations[a]	1	2	3	4	5
Plants with dry branches[b]	0	2	3	3	3

a) Every 2 days, 20 ml of C. illinoinensis aqueous extract for each plant; b) Total number of plants in test group n=10

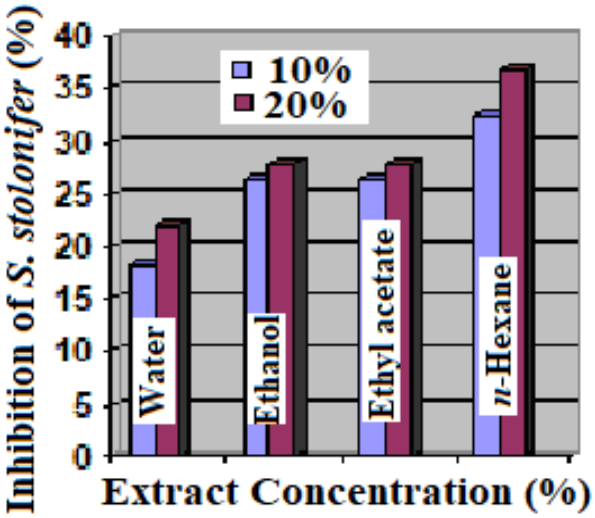


Figure 4: Inhibition (%) of R. stolonifer by extracts of *P. farcta*

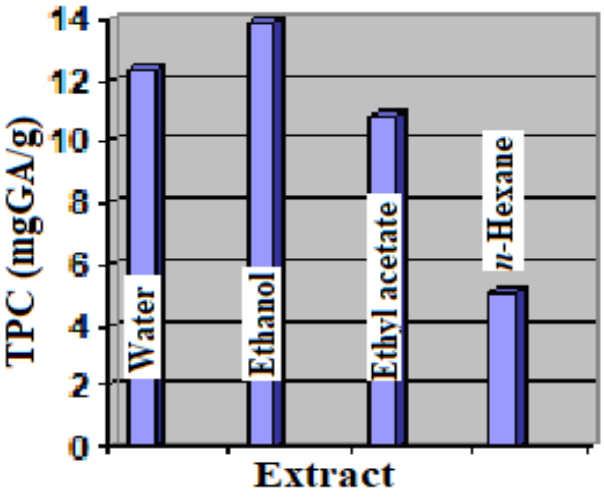


Figure 5. Total phenolic content of extracts of P. farcta (mg gallic acid in 1 g of dry extract)

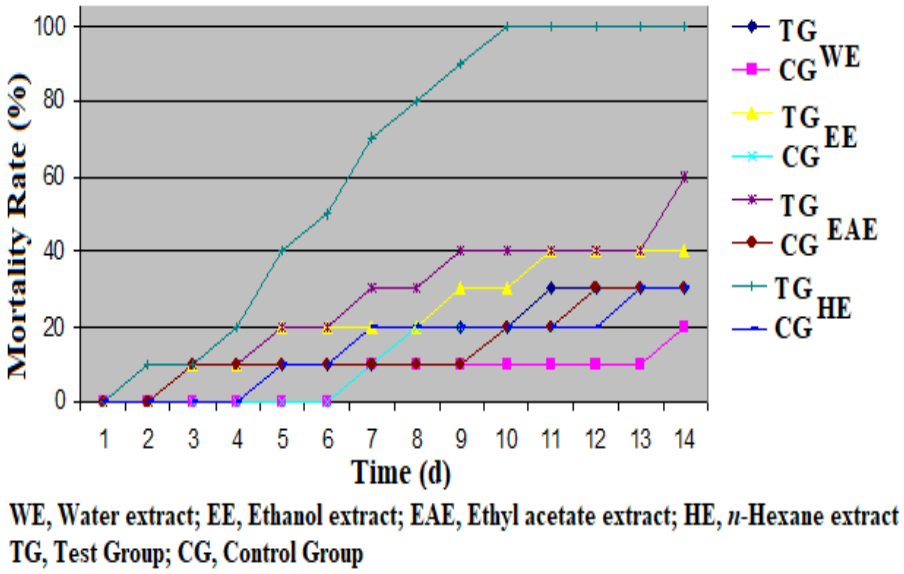


Figure 6. The mortality rate of termites (*K. sinaicus*) as a result of feeding extracts loaded paper

Anti-termite activity

Anti-termite activity was measured over 4 weeks. Results presented here are by day, where the mortality rate of termites (%) is presented in tested and control groups. Table 6 and Figure 6.

Biocontrol of *P. farcta* with *C. illinoinensis* aqueous extract

After irrigation of 10 plants of *P. farcta* with 20 ml of *C. illinoinensis* aqueous extract every 2 days (control irrigated with water), none of them died. But after the fourth day (second irrigation with extract), some of the test group plants started showing dry branches. The control group plants continued growing normally with no dry branches. Results are summarized in Table 7 and Figure 7.

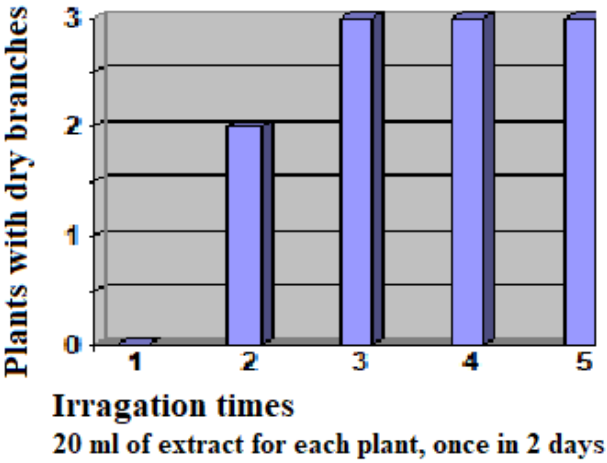


Figure 7. *P. farcta* with dry branches after irrigation with aqueous extract C. illinoinensis

Discussion

In this research, we studied some of the medicinal properties of *P. fracta*, that in addition to being a plant with high medicinal and other practical potentials, it is also a very widespread weed that harms agricultural fields. It is an invasive species not only in locations very far away of its natural habitat, but it also invaded parts of Western of Asia where it did not grow in the past.[77] So, in addition to studying some of it's not reported medicinal properties, one of the objectives of this research was to develop a biocontrol method against it. Two considerations were taken into account. First, the use of synthetic chemicals as herbicides has many adverse health and environmental effects. An excellent example of this can be glyphosate (Figure 8 A).[78]

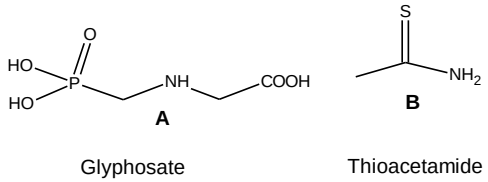


Figure 8. Structures of Glyphosate (A) and thioacetamide (B)

The second consideration is that use of plants extracts as herbicides is already known, especially of C. illinoinensis aqueous extract, that we used.[79] But success in this part was very limited: none of the tested group plants died. Some of them partially dried but they lived and a few weeks after treatment, they were all green again. This means that many more tests of this method are needed, but we also plan to use extracts of other plants, known for their toxicity.

As we have presented, medicinal and other properties of *P. farcta* are being published continuously. For example, in addition to testing its potential as an antioxidant according

to classical methods (DPPH, FRAP, ABTS, etc.), some interesting studies are published. M. Zehab et al. tested the activity of seed hydroalcoholic (50 %) against oxidative stress that was orally induced in rats by thioacetamide (Figure 8 B).[80]

One of the most interesting reports that were recently published, examines the concentration of sesamin in *Cuscuta palaestina*, a parasitic plant that grows over other plants, such as *P. Farcta*.[81] Among five very common plants (near east) host plants (*P. farcta*, *Portulaca oleracea*, *Corchorus olitorius*, *Malva sylvestris* and *Cichorium intybus*), *C. Palaestina* that grew on *P. farcta* contained the highest concentration of sesamin: 8.45 ppm. Despite this, researchers proved that sesamin is produced by the parasitic plant and not transferred to it by the hosting plant.

Published reports of the different properties of P. farcta are mostly consistent, and in these cases, we did not examine these reports. This was not the case of the total phenolic content (TPC). A. Molan et al.,[25] reported 17.3 mg of gallic acid equivalent in 1 g of dry extract, while E. Karimi et al. reported 24.2.[26] This is not clear since both groups reported these results for methanolic extract. Our results are lower than both reports, 13.4 mg, but we tested ethanolic extract, which is expected not to be meaningfully different.

Toxicity of *P. farcta* to humans is still not clear. The only evident reported case was published in Turkey, where children (3.5-6 years old) consumed seeds with pods.[82] No other reports were published (or known) before or after this case. On the contrary, aerial parts of the plant can be used in many ways for wound healing as can be seen in the concise review article of Bahmani and Asadi-Samani.[83] But despite this, we reported here that extracts of aerial parts could be toxic to fungi and termites. For anti-termite activity, several efficient and facile methods were published and we found the method of O. Ndukwe best,[76] even though, A. Alshehry's method is also very useful.[84]

Conclusions

A- Many of the medicinal properties of P. farcta were investigated, but others were very partially studied (anticancer) or did not (antiobesity, anti-nervous system disorders).

B- The complete chemical composition of the plant is not known, and there is an urgent need for this, to promote drug discovery and other applications.

C- We reported good results of antifungal activity of the plant extracts. This research needs more studying in order to identify the active natural products responsible for this activity.

D- Our findings of anti-termite activity are very encouraging. Further research is needed.

E- n-Hexane extract proved very active. Special attention must be paid to this fact.

F- Biocontrol of P. farcta is still a very challenging subject since this plant is a weed that harms wide agricultural areas. Intensive additional research must be done.

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Received: 09.12.2018.
Accepted: 29.01.2019.

[BMIM]OH MEDIATED NEW SYNTHESIS 3-(1H-INDOL-3-YL)ACRYLONITRILE DERIVATIVES

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Keywords: Ionic liquid, [Bmim]OH and green synthesis

[Bmim]OH mediated new synthesis of 3-(1H-indol-3-yl)acrylonitrile derivatives **6** have been developed by the reaction of diethyl phthalate (**1**) with ethyl cyanoacetic acid hydrazide (**2**) to form 3-(1,4-dioxo-3,4-dihydrophthalazin-(1H)-yl)-3-oxopropanenitrile (**3**). Then compound **3** reacted with indole-3-aldehyde (**4)** by Knoevenagel condensation to form compound 2-(1,4-dioxo-1,2,3,4-tetrahydrophthalazine-2-carbonyl)-3-(1H-indol-3-yl)acrylonitriles (**5**). Compounds **5** undergo alkylation with alkylating agents to form **6** with good yields. Compounds **6** could also be synthesized by alkylation of **4** followed by condensation with **3**.

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INTRODUCTION

Ionic liquids as catalysts[1] and /or media[2] in reactions have been widely used in organic transformations due to their advantages such as good solvating ability, negligible vapor pressure, high polarity and ease of work-up. [Bmim]OH (1-butyl-3-methylimidazolium hydroxide) is one such task-specified ionic liquid which acts as reaction medium as well as a basic catalyst and has got various applications[3] in the field of synthetic methodology development.

Heterocycles containing the phthalazine moiety are of interest because they show some pharmacological and biological activities.[4-5] Mogilaiah et al[6] reported the synthesis of 1,8-naphthyridine-3-carbonylphthalazine-1,4-diones by the condensation of 1,8-naphthyridine-3-carboxylic acid hydrazides with phthalic anhydride using p-toluene sulphonic acid (PTSA) as a catalyst under solid state conditions. Mogilaiah et al[7] also reported the microwave irradiation of a mixture of 3-aryl-2-hydrazino-1,8-naphthyridines with phthalic anhydride in the presence of a catalytic amount of DMF resulting in 2-(3-aryl-1,8-naphthyridin-2-yl)-1,2,3,4-tetrahydrophthalazine-1,4-diones.

Indole derivatives continue to receive much attention in organic synthesis because of their biological activities.[8,9] Among them, 3-substituted indole is one of the 'privileged medicinal scaffold,' found in many biologically active compounds and natural products.[10,11] Through appropriate functional group modifications, these scaffolds are capable of providing ligands for a number of functionally and structurally discrete biological receptors. 3-Substituted indole scaffolds are found in a number of biologically active compounds especially with anticancer, anti-tumor,[12] hypoglycaemic, anti-inflammatory, analgesic and antipyretic activities.[13-16]

Keeping in view the potential importance of the phthalazine and indole ring containing compounds, we now wish to report our studies on reactions of phthalic anhydride with hydrazide derivatives and their further modifications.

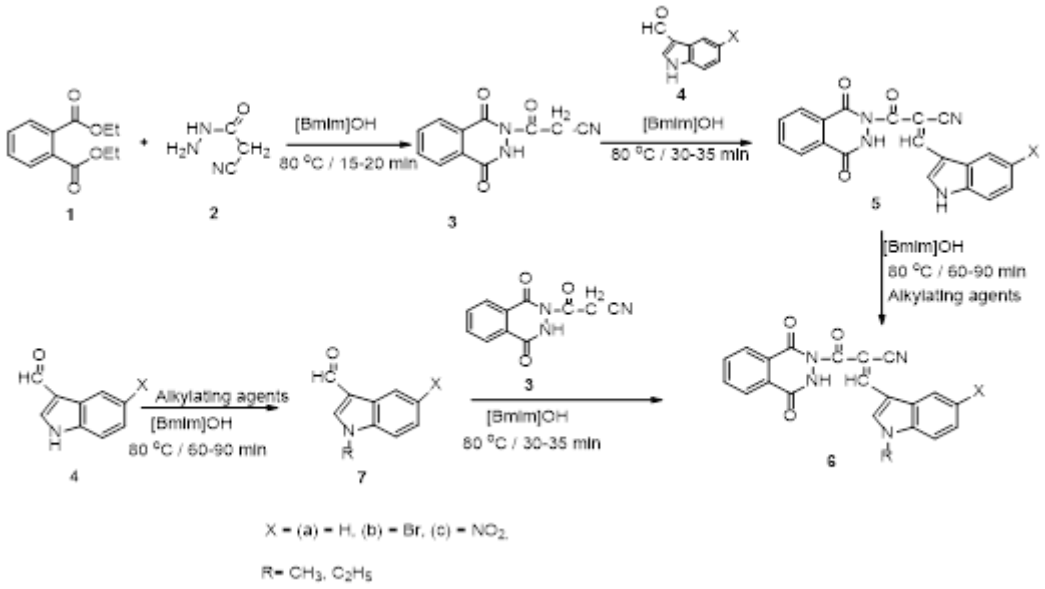
RESULTS AND DISCUSSION

At first, we have developed the condensation of diethyl phthalate (**1)** (1 mmol), ethyl cyanoacetic acid hydrazide[8] (**2**) (1 mmol) to from 3-(1,4-dioxo-3,4-dihydrophthalazin-(1H)-yl)-3-oxopropanenitrile[17] (**3**) in the presence of 1-ethyl-3-methylimidazolium hydroxide solution [Bmim]OH (5 ml) at 80 °C for 15-20 min. Compound **3** (1 mmol) was reacted with indole-3-aldehyde (**4a**) (1 mmol) in the presence of [Bmim]OH (5 ml) at 80 °C for 30-35 min to form 2-(1,4-dioxo-1,2,3,4-tetrahydrophthalazine-2-carbonyl)-3-(1H-indol-3-yl)acrylonitriles (**5a**) in 87 % yield on simple work-up of reaction mixture (TABLE 1, entry 1).

The gross structure of this product was assigned on the basis of its spectral data. Furthermore, the compound was supposed to be E-configuration on the presumption that bulky groups in a trans position would confer thermal stability on the molecule. This has been found to be the case by a careful examination of the Frame-work molecular models of both E and Z - configurations of **5a** wherein it was observed that there was a minimum number of steric interactions in the E – configuration.

The latter on alkylation of **5a** with an alkylating agent, i.e. dimethylsulfate (DMS) in the presence of [Bmim]OH (5 ml) at 80 °C for 60-90 min gave the corresponding indole-NH-alkylated derivatives **6a** with 85 % yield.

The above reaction was examined by carrying out the condensation of **3** with **4a** in the presence of different ionic liquids ([Bmim]Br, [Bmim]BF_4 and [Bmim]$AlCl_4$)) at different temperature (TABLE 1). However, the condensation of **3** with **4a** using [Bmim]OH as a catalyst and solvent media at 80 °C for 30 min gave the best yield (87 %) of **5a**. The alkylation reaction was also optimized by carrying out the alkylation of **5a** with DMS in the presence of different ionic liquids ([Bmim]Br, [Bmim]BF_4 and [Bmim]$AlCl_4$)) at different temperature (TABLE 1). However, the alkylation of **5a** with methyl bromide using [Bmim]OH as a catalyst and solvent media at 80 °C for 60 min gave the best yield (85 %) of **6a**.



Scheme 1. Synthesis of **6a-6f**

Table 1. Effect of ionic liquid and temperature on the reaction of **3** with **4a** yielding **5a**.

Ionic liquid	Temperature, °C	Time, min	Yield, %
[Bmim]OH	80	30	87
[Bmim]Br	80	45	72
[Bmim]BF_4	80	50	63
[Bmim]$AlCl_4$	80	60	54
[Bmim]OH	100	30	70
[Bmim]Br	100	35	62
[Bmim]BF_4	100	40	61
Bmim]$AlCl_4$	100	45	50
[Bmim]OH	RT	120	84
[Bmim]Br	RT	180	82
[Bmim]BF_4	RT	190	75
[Bmim]$AlCl_4$	RT	200	70

Table 2. Effect of ionic liquid & temperature on the reaction of **5a** with dimethyl sulfate yielding **6a**.

Ionic liquid	Temperature, °C	Time, min	Yield, %
[Bmim]OH	80	60	85
[Bmim]Br	80	75	80
[Bmim]BF_4	80	70	82
[Bmim]$AlCl_4$	80	90	80
[Bmim]OH	100	45	75
[Bmim]Br	100	55	73
[Bmim]BF_4	100	65	70
Bmim]$AlCl_4$	100	65	65
[Bmim]OH	RT	240	83
[Bmim]Br	RT	360	82
[Bmim]BF_4	RT	360	81
[Bmim]$AlCl_4$	RT	350	80

After having optimized the reaction conditions, the generality of the reaction was confirmed by carrying out the condensation of 3 with 4a-4c in the presence of [Bmim]OH at 80 °C for 30-35 min gave the corresponding compounds **5a-5c** in good yields. The latter on alkylation of **5a-5c** with alkylating agents in the presence of [Bmim]OH at 80 °C for 60-90 min gave the corresponding indole-NH-alkylated derivatives **6a-6f**. Using this strategy, alternatively, **6a-6f** were prepared by alkylation of **4a-4c** with alkylating agents in the presence of [Bmim]OH at 80 °C for 60-90 min to form **7a-7f** followed by Knoevenagel condensation of the initial product with **3** in the presence of [Bmim]OH at 80 °C for 30-40 min. All of the above reactions are summarized in Scheme 1.

EXPERIMENTAL SECTION

Melting points are uncorrected and were determined in open capillary tubes in a sulphuric acid bath. TLC was run on silica gel–G and visualization were done using iodine or UV light. IR spectra were recorded using Perkin–Elmer 1000 instrument in KBr pellets. 1H NMR spectra were recorded in DMSO–d6 using TMS as internal standard using 400 MHz spectrometer. Mass spectra were recorded on an Agilent-LCMS instrument. Starting materials **1** and **4a-4c** were obtained from commercial sources and used as such.

Preparation of 3

A mixture of **1** (10 mmol), **2** (10 mmol) and [Bmim]OH (20 mL) was heated at 80 °C along with stirring and maintained until the completion of reaction as checked by TLC (15-20 min). To the resulting oily reaction mixture was added ethanol (30 mL) to force out the crude product from the polar ionic liquid reaction medium.

The separated solid mass was collected by filtration and dried in oven to obtain crude **3**. The later, were recrystallized from ethanol solvent to get the pure **3**.

3: M.p. 151–153 °C; IR (KBr)17 : 3293-3518 cm-**1** (broad, medium, -NH-), 1748 cm^{-1} (sharp, strong, -CO-), 1682 cm^{-1} (sharp, strong, -CO- of amide group), 1614 cm^{-1} (sharp, strong, -CO- of amide group); ^{1}H-NMR (DMSO-d_6, 400 MHz) : δ 4.2 (s, 2H, CH_2), 7.9-8.0 (m, 4H, Ar-H), 11.1 (s, 1H, -NH, D_2O exchangeable); 13C-NMR (DMSO-d_6, 400 MHz): δ 31.6, 36.9, 114.8, 124.8, 131.1, 136.2, 164.1, 164.8, 166.1; M^++1 = 230.

Preparation of 5 from 3 and 4

A mixture of **3** (10 mmol), 4 (10 mmol) and [Bmim]OH (20 mL) was heated at 80 °C along with stirring and maintained until the completion of reaction as checked by TLC (35-40 min). To the resulting oily reaction mixture was added ethanol (30 ml) to force out the crude product from the polar ionic liquid reaction medium. The separated solid mass was collected by filtration and dried in oven to obtain crude **5**. The later, were recrystallized from ethanol solvent to get the pure **5**.

5a: M.p.: >220 °C; IR (KBr) 3017-3165 cm^{-1} (broad, medium, -NH-), 1661 cm^{-1} (sharp, strong, -CO- of amide group), 1600 cm^{-1} (sharp, strong, -CO-); ^{1}H- NMR (DMSO-d_6, 400 MHz) : 7.2-8.6 (m, 10H, Ar-H and C=CH), 11.1 (s, 1H, -NH, D_2O exchangeable), 12.6 (s, 1H, -NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 92.8, 109.8, 112.9, 117.8, 118.4, 122.0, 123.6, 123.8, 127.1, 129.3, 132.1, 135.3, 136.1, 144.8, 162.0, 165.1; M^++1 = 357.

5b: M.p.: >220 °C; IR (KBr) 3012-3264 cm-1 (broad, medium, -NH-), 1668 cm-1 (sharp, strong, -CO- of amide group), 1605 cm-1 (sharp, strong, -CO-); 1H-NMR (DMSO-d_6, 400 MHz) : 7.4-8.6 (m, 9H, Ar-H and C=CH), 11.2 (s, 1H, -NH, D_2O exchangeable), 12.4 (s, 1H, -NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 92.6, 108.3, 111.4, 115.3, 117.3, 122.3, 123.4, 123.9, 127.3, 127.3, 130.3, 136.3, 138.3, 143.2, 161.4, 164.2; M^++1 = 435.

5c: M.p.: >220 °C; IR (KBr) 3016-3232 cm^{-1} (broad, medium, -NH-), 1670 cm^{-1} (sharp, strong, -CO- of amide group), 1610 cm^{-1} (sharp, strong, -CO-); ^{1}H-NMR (DMSO-d_6, 400 MHz) : 7.1-8.6 (m, 9H, Ar-H and C=CH), 11.3 (s, 1H, -NH, D_2O exchangeable), 12.4 (s, 1H, -NH, D2O exchangeable); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 91.3, 108.3, 111.5, 115.4, 118.3, 121.4, 123.5, 123.7, 124.2, 129.5, 132.3, 134.3, 135.2, 141.5, 160.1, 164.2; M^++1 = 402.

Preparation of 6 from 5 and alkylating agents

A mixture of **5** (10 mmol), alkylating agent (10 mmol) and [Bmim]OH (20 mL) was heated at 80 °C along with stirring and maintained until the completion of reaction as checked by TLC (60-90 min). To the resulting oily reaction mixture was added ethanol (30 ml) to force out the crude product from the polar ionic liquid reaction medium. The separated solid mass was collected by filtration and dried in oven to obtain crude **6**. The later, were recrystallized from ethanol solvent to get the pure **6**.

6a: M.p.: >230 °C; IR (KBr): 3316-3374 cm^{-1} (broad, medium, -NH-), 1658 cm^{-1} (sharp, strong, -CO- of amide group), 1601 cm^{-1} (sharp, strong, -CO-); ^{1}H-NMR (DMSO-d6, 400 MHz) : 4.0 (s, 3H, CH_3), 7.3-8.6 (m, 10H, Ar-H & C=CH), 11.1 (s, 1H, -NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 33.8, 92.5, 108.8, 111.4, 117.6, 118.5, 122.4, 123.6, 123.8, 127.6, 129.3, 135.2, 135.3, 136.8, 144.0, 162.0, 165.1; M^++1 = 371.

6b: M.p.: >230 °C; IR (KBr): 3310-3390 cm^{-1} (broad, medium, -NH-), 1650 cm^{-1} (sharp, strong, -CO- of amide group), 1610 cm^{-1} (sharp, strong, -CO-); ^{1}H-NMR (DMSO-d_6, 400 MHz) : 4.1 (m, 2H, CH_2), 2.2 (t, 3H, CH_3), 7.3-8.4 (m, 10H, Ar-H and C=CH), 11.1 (s, 1H, -NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 23.0, 34.6, 90.3, 104.4, 110.3, 114.6, 115.6, 120.3, 122.4, 124.4, 125.2, 128.2, 134.5, 135.5, 136.7, 143.5, 161.3, 164.4; M^++1 = 385.

6c: M.p.: >230 °C; IR (KBr) : 3315-3379 cm^{-1} (broad, medium, -NH-), 1653 cm^{-1} (sharp, strong, -CO- of amide group), 1604 cm^{-1} (sharp, strong, -CO-); ^{1}H-NMR (DMSO-d_6, 400 MHz) : 3.9 (s, 3H, CH_3), 7.3-8.6 (m, 9H, Ar-H and C=CH), 11.0 (s, 1H, -NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 33.2, 91.3, 107.3, 110.3, 114.3, 116.6, 121.3, 122.4, 123.7, 127.5, 129.4, 134.1, 135.2, 136.7, 144.1, 162.1, 165.3; M^++1 = 448.

6d: M.p.: >230 °C; IR (KBr) : 3312-3394 cm^{-1} (broad, medium, -NH-), 1678 cm^{-1} (sharp, strong, -CO- of amide group), 1621 cm^{-1} (sharp, strong, -CO-);1H- NMR (DMSO-d6, 400 MHz) : 4.0 (m, 2H, CH_2), 2.1 (t, 3H, CH_3), 7.3-8.6 (m, 9H, Ar-H and C=CH), 11.3 (s, 1H, -NH, D_2O exchangeable ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 23.2, 34.7, 91.2, 103.4, 109.2, 112.1, 114.5, 121.2, 122.3, 123.5, 125.3, 128.5, 133.1, 134.2, 135.2, 142.2, 161.9, 163.8; M^++1 = 462.

6e: M.p.: >230 °C; IR (KBr) : 3313-3379 cm^{-1} (broad, medium, -NH-), 1659 cm^{-1} (sharp, strong, -CO- of amide group), 1600 cm-1 (sharp, strong, -CO-); 1H-NMR (DMSO-d_6, 400 MHz) : 4.1 (s, 3H, CH_3), 7.2-8.9 (m, 9H, Ar-H and C=CH), 11.2 (s, 1H, -NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, 400 MHz): δ 32.8, 91.5, 105.7, 110.3, 114.9, 118.4, 121.3, 122.4, 126.3, 127.5, 127.8, 134.1, 134.6, 135.9, 143.2, 162.0, 165.4; M^++1 = 416.

6f: M.p.: >230 °C; IR (KBr) : 3286-3379 cm^{-1} (broad, medium, -NH-), 1659 cm^{-1} (sharp, strong, -CO- of amide group), 1611 cm-1 (sharp, strong, -CO-); ^{1}H- NMR (DMSO-d_6, 400 MHz) : 4.3 (m, 2H, CH_2), 2.3 (t, 3H, CH_3), 7.3-8.5 (m, 9H, Ar-H and C=CH), 11.1 (s, 1H, -NH, D_2O exchangeable); 13C-NMR (DMSO-d6, 400 MHz): δ 23.3, 34.4, 91.4, 104.5, 111.4, 114.5, 114.9, 121.5, 121.4, 123.4, 124.2, 125.7, 133.1, 135.3, 135.8, 142.4, 161.2, 164.9; M^++1 = 430.

Preparation of 7a-7f from 4a-4c and alkylating agents

A mixture of **4** (10 mmol), alkylating agents (10 mmol) and [Bmim]OH (20 mL) was heated at 80 °C along with stirring and maintained until the completion of reaction as checked by TLC (60-90 min). To the resulting oily reaction mixture was added ethanol (30 mL) to force out the crude product from the polar ionic liquid reaction medium.

The separated solid mass was collected by filtration and dried in an oven to obtain crude **7**. The later was recrystallized from ethanol solvent to get the pure **7**.

Preparation of 6a-6f from 7a-7f & 3

A mixture of **7** (10 mmol), **3** (10 mmol) and [Bmim]OH (20 mL) was heated at 80 °C along with stirring and maintained until the completion of reaction as checked by TLC (35-40 min). To the resulting oily reaction mixture was added ethanol (30 mL) to force out the crude product from the polar ionic liquid reaction medium. The separated solid mass was collected by filtration and dried in an oven to obtain crude **6**. The later was recrystallized from ethanol solvent to get the pure **6**.

CONCLUSION

In summary, we have successfully developed syntheses of new 2-(1,4-dioxo-1,2,3,4-tetrahydrophthalazine-2-carbonyl)-3-(1H-indol-3-yl)acrylonitriles (**6**) in two different routes under green conditions using ionic liquid without formation of any by-products with good yields.

Acknowledgment

The authors would like to thank Mewar University, Rajasthan for permitting the research work and for constant encouragement.

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Received: 02.01.2019.
Accepted: 07.02.1019.

ELECTROCHEMICAL INHIBITION BIOSENSOR ARRAY FOR RAPID DETECTION OF WATER POLLUTIONS BASED ON BACTERIA IMMOBILIZED ON SCREEN-PRINTED GOLD ELECTRODES

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Keywords: Water pollution, inhibition biosensor, bacteria-based biosensor, immobilized bacteria, electrochemical sensor, sensor array, pattern recognition.

This work reports on the development of a bacteria-based inhibition biosensor array for detection of different types of pollutions, i.e. heavy metal ions (Zn^{2+}), pesticides (DDVP) and petro-chemicals (pentane), in water. The biosensor chip for preliminary identification of the above water pollutants is based on three types of bacteria (*Escherichia coli*, *Shewanella oneidensis* and *Methylosinus trichosporium* OB3b) immobilized on screen-printed gold electrode surface via poly L-lysine which provides strong adhesion of bacterial monolayer to the electrode without losses of biological function. A series of optical measurements and DC electrochemical measurements were carried out on these three types of bacteria species immobilized on modified screen printed gold electrodes as well as on the bacteria in solution samples. The principle of electrochemical detection of pollutants is based on the facts that live bacteria adsorbed (or immobilized) on the electrode surface appeared to be insulating and thus reducing the electrochemical current, while the bacteria damaged by pollutants are less insulating. The results obtained demonstrated different effects of the three different types of analytes studied, e.g. Zn^{2+}, DDVP, and pentane, on the three bacteria used. The findings are encouraging for application of a pattern recognition approach for identification pollutants which may lead to development of a novel, simple, and cost-effective bio-sensing array for preliminary detection of environmental pollutants in water.

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Introduction

Extensive industrial and agricultural activities have contaminated the environment with large number of toxic chemicals, particularly, heavy metals, pesticides and petro-chemicals which are spread in the atmosphere and aquatic environment, and have negative impacts on all the living organisms.[1] Water Pollution has been described as any natural or human-made release of the chemical, biological, or radioactive elements to the aquatic environment which affect the health and wellbeing of the living species in water resources, and pose a serious threat to human, animals, plants, and microorganisms.[2] Such pollutants might cause either major destruction with direct visible effects on the environment, or minor destruction in the system of living organisms' life cycle due to disturbance of a delicate biological balance which becomes noticeable after a certain time.[3] The main sources of heavy metal contamination are associated with mining, manufacturing and chemical industries, and transport,[4] while pesticides have been released into the environment as a result of extensive agriculture,[5] and petro-chemical industry and transport heavily contribute to environmental pollution with a wide range of chemicals ranging from relatively harmless hydrocarbons, alcohols, and ketones to much more dangerous benzene derivatives (BTEX).[6] This study focuses on detection of three chemicals belonging to different classes of contaminants, i.e. Zn^{2+} ions, DDVP pesticide and pentane.

Great deal of effort has been made to develop technologies for monitoring pollutants in aqueous environment, because the evaluation of these contaminants is considered as one of the most serious current global problems.[7] A number of analytical methods, such as atomic absorption or atomic emission spectroscopies (AAS, AES), inductively coupled plasma mass spectroscopy (ICP-MS), cold vapour atomic fluorescence spectroscopy (CVAFS), and high pressure liquid chromatography (HPLC) are capable of detecting traces of toxic pollutants,[15,16] however these methods require sophisticated analytical equipment, specialised laboratories and highly qualified personnel, which make such analysis very expensive and time consuming. Therefore, the development of alternative detection technologies, for example, simple and inexpensive biosensor devices, capable of rapid detection of environmental pollutions, is urgently needed.[10]

A particular interest is in development of the inhibition type of bio-sensors which do not detect pollutants directly, but through the monitoring changes in functionality of bio-receptors caused by exposure to pollutants.[11] Such inhibition sensors (or rather senor arrays) are quite versatile and applicable for detection of various chemicals using a limited set of bio-receptors. The sensitivity and selectivity of these sensors may not be great but sufficient for quick detection and identification of suspected samples for further more detailed analysis; the cost and time of analysis can be substantially reduced as a result.

Enzymes are typically used as bio-receptors in inhibitions sensors.[12] The drawback of such enzyme-based inhibition sensors is in poor stability of enzyme.[13] Several inhibition

biosensors based on whole cells have been reported in the last two decades, which mostly use optical transduction techniques such as fluorescence.[14] However, these approaches rely heavily on genetically modified strains (e.g. strains with green fluorescent protein under the control of specific promoters), and hence have disadvantages of complexity and high cost.[15,16] There have also been reports on electrochemical sensors based on whole-cells.[17,18] Bacteria could be versatile bio-receptors for traces of toxic environmental pollutants because these molecules or ions interfere with biological processes (e.g. catabolism and photosynthesis) and modify bacterial activity that can be monitored in a number of ways.[19] It is relatively easy to immobilize whole bacterial cells on electrodes and electrochemically monitor redox species which participate in the cells' metabolic processes, e.g., oxygen, hydrogen peroxide or protons.[20]

In recent years, a number of microbial biosensor systems based on different transducing methods have been developed for environmental, food, and biomedical applications including the detection of various inorganic and organic pollutants in water, such as heavy metal ions, pesticides, hydrocarbons, etc.[21,22] Similar to this study, electrochemical detection principles have been exploited in the study of inhibition effects of environmental pollutants on *E. coli* bacteria.[23,24]

In our previous study of optical and electrical properties of solutions of two types of bacteria (*E. coli* and *Deinococcusr adiodurans*), a correlation between the optical density and electrical conductivity and the bacteria concentration in liquid samples was established, and then utilized for identification of two types of pollutants, e.g. heavy metals and radionuclides, by their inhibition effects on the above bacteria.[25] This approach was further developed recently.[26] This work has focused mostly on electrochemical detection of heavy metal ions (Hg^{2+}) using two types of bacteria (*E. coli* and *Shewanella oneidensis*) which were either free in solution or immobilized on the electrode surface. In the current work, we went further by expanding the bacterial sensor array (three types of bacteria were used *E. coli*, *S. oneidensis*, and *Methylosinus trichosporium* OB3b) as well as the range of pollutants tested (Zn^{2+} ions, DDVP, and pentane which belong to different classes of chemicals). The main aim of this work was to develop the inhibition bacterial sensor array for detection of different types of water pollutants using pattern recognition principles. The results of this work may lead to development of a novel, simple and cost-effective bio-sensing technology for preliminary detection (screening) of water pollutants.

Experimental methodologies

Preparation of bacteria samples.

Three diverse bacterial strains were selected for this work: (i) the model Gram-negative *E. coli* K12, known to be sensitive to various types of pollutants including heavy metals, pesticides, and hydrocarbons,[27] (ii) *S. oneidensis* MR-1, a Gram-negative bacterium known to tolerate and interact with heavy metals[28] and (iii) the methanotrophic bacterium *M. trichosporium* (OB3b) strain (a Gram-negative bacterium that grows on methane and is also able to co-oxidise a range of other hydrocarbons and hydrophobic organic molecules.[29,30] *E. coli* cultures were grown at 37 °C for 16 h in LB broth and LB agar.[31] *S. oniedensis* cultures were grown at 30 °C for 24 h in the same medium,[32] while *M. trichosporium* OB3b was grown at 30 °C for 2 weeks in flask cultures in nitrate mineral salts (NMS) medium and on NMS agar plates, using methane as the carbon source, as described previously.[33]

The surface of screen-printed gold electrodes was modified with a (0.1%) solution of poly L-lysine (PLl; Sigma-Aldrich; 0.1 mg/ml in deionised water) for 1 h at 37 °C. The bacteria were immobilized by pippeting a liquid culture at a stationary phase of the appropriate strain onto the electrode and keeping it in contact with the surface for 1 h, then washing off non-bound bacteria with phosphate buffered saline (Sigma-Aldrich 14200-067).

Preparation of analytes

The samples of $ZnCl_2$, DDVP and pentane (all from Sigma-Aldrich) were prepared at concentrations of 0.1, 1, 10, 100 mM by consecutive dilution of 1 M stock solutions in deionised water. The stock solution of pentane was prepared in a 40% (v:v) ethanol:water mixture. Liquid bacteria culture samples were mixed with these solutions in 1:1 ratio and incubated for 2 h at 22-23 °C. The samples of immobilized bacteria were treated similarly by immersing the electrode functionalized with bacteria into the required solution of pollutants for 2h.[26]

Optical and SEM characterization of bacteria

The effect of the above pollutants on the bacterial cultures was examined using three different optical experimental techniques: fluorescence microscopy, UV–visible spectrophotometry, and flow cytometry. A Becton-Dickinson FACS Calibur flow cytometer was used to count live and dead bacteria after staining them with the BacLight live/dead bacterial viability kit (Molecular Probes). Fluorescence microscopy of liquid cultures and bacteria immobilized on the screen printed gold electrodes was performed using an Olympus-BX60 instrument using liquid bacterial samples also stained with the BacLight kit.[34,35] Optical density of bacterial cultures with measured at 600 nm with a 6715 UV/Vis spectrophotometer (Jenway). For scanning electron microscopy (SEM) immobilized bacteria were fixed on double-sticking carbon tape mounted on a sample holder and coated with a few-nanometer-thick layer of carbon using a carbon evaporator (Edwards E306A; Edwards, United Kingdom). The scanning electron microscopy using in all experiments is FEI-Nova SEM. The system was operated at 1 to 15 kV for high-resolution secondary electron imaging and elemental analysis.

Electrochemical measurements

Cyclic voltammograms (CVs) were recorded in a voltage range from -0.5 V +0.5 V using DropSens gold screen-printed three-electrode assemblies (which include Ag/AgCl reference electrode) and a DropSens microSTAT4000P potentiostat. CVs of liquid bacterial cultures were recorded

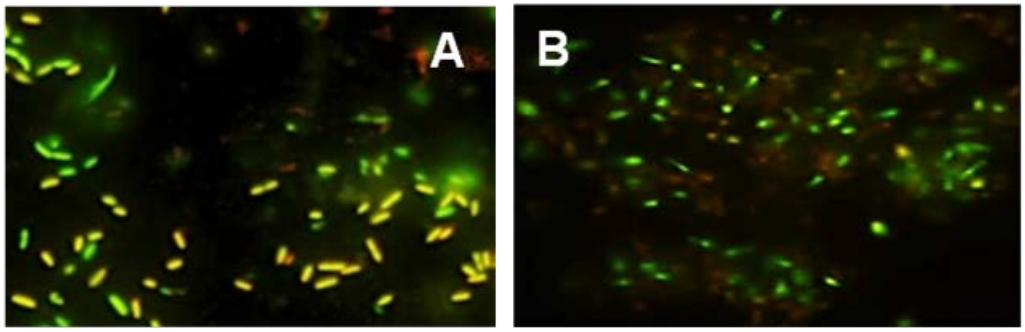

Figure 1. Fluorescence microscopy images of immobilized *Shewanella oneidensis* before (A) and after (B) treatment with $ZnCl_2$ salt (1 mol).

on screen-printed electrodes immersed into the bacterial suspension. Measurements were taken on liquid samples of all three bacteria before and after treatment with each pollutant at each concentration. The CV measurements of the electrodes with freshly immobilized bacteria were carried out in LB broth before and after treatment with each pollutant at each concentration.

Results and Discussion

Optical characterization

Fluorescence microscopy images in Figure 1 show the effect 1M solution of $ZnCl_2$ on *Shewanella oneidensis* bacteria immobilized on screen printed gold electrodes and treated with the BacLight live/dead stain.[36] The exposure to Zn^{2+} ions reduced the number of live bacteria (green or yellow) and increases the dead ones (red or orange). Similar experiments were carried out for all three types of bacteria and for all analytes used, and the resulted counts of live (green or yellow) and dead (red or orang) bacteria on recorded images of identical dimensions are summarized in Table 1.

Table 1. The numbers of live and dead bacteria immobilized on modified screen printed gold electrodes for all three bacteria before and after treatment with 1M solutions of the three pollutants for 2 h.

Bacteria	Pollu-tants	Before exposure		After exposure	
		Live	Dead	Live	Dead
E. coli	$ZnCl_2$	75	19	21	65
S. oneidensis	$ZnCl_2$	57	11	52	28
M. trichosporium	$ZnCl_2$	69	21	34	79
E. coli	DDVP	62	17	31	73
S. oneidensis	DDVP	79	18	25	41
M. trichosporium	DDVP	81	25	23	65
E. coli	Pentane	43	13	19	51
S. oneidensis	Pentane	49	22	38	17
M. trichosporium	Pentane	93	20	62	14

These data revealed that *E. coli* and *M. trichosporium* (OB3b) more severely affected by large concentrations of Zn^{2+} ions than *S. oneidensis*. The negative effect of DDVP is dramatic and more or less similar for all three bacteria. Pentane, however, did not affect *M. trichosporium* (OB3b) strain, though it reduced the viability of both *E. coli* and *S. oneidensis*. The pattern of responses of immobilized bacteria to the above pollutants is similar to what was previously observed for the same bacteria strains in solution.[26] The results of optical density (OD_{600}) study of liquid bacteria samples in Figure 2 shows the effect of exposure to a high concentration (1 M) of $ZnCl_2$. The results were similar to those obtained via fluorescent microscopy i.e., all bacteria appeared to be affected by $ZnCl_2$ though this effect was less pronounced for *S. oneidensis*. It is important to state that the optical density measurements, which are based on light scattering, could be affected by different motility of the bacteria studied, whereas this would not affect the bacteria held in place on the gold electrode surface. Similarly, death of bacteria without lysis or morphological change would not contribute to a change in OD.

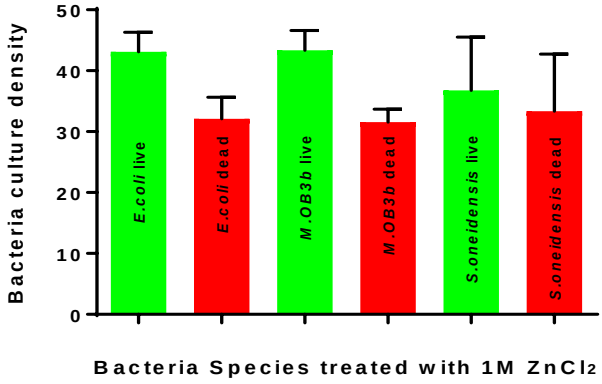

Figure 2. Bacteria culture concentration before and after treatment with large concentrations (1 M) of $ZnCl_2$.

Flow cytometry measurements combine the advantages of fluorescence microscopy and optical counting of individual cells. Typical results of flow cytometry for cultures of all three bacteria before and after treatment with a 1M solution of $ZnCl_2$ for 2 h and subsequent staining with the BacLight are presented in figure 3. The increase in the counts of dead bacteria after exposure to $ZnCl_2$ is apparent for all three types of bacteria studied. In addition to that, after $ZnCl_2$ treatment, dead *E. coli* and *M. trichosporium* (OB3b) bacteria appear mostly in bottom-left quadrant of the graph in Figure 3C and 3B indicating the increase in the bacteria size most-likely due to the enlargement or rapture of cell walls. *S. oneidensis* was less affected by $ZnCl_2$ as one can see in Figure 3A. Flow cytometry tests were carried out for the other two pollutants, e.g. DDVP and pentane, and the results were summarized in table 2 as the percentage of live and dead bacteria.

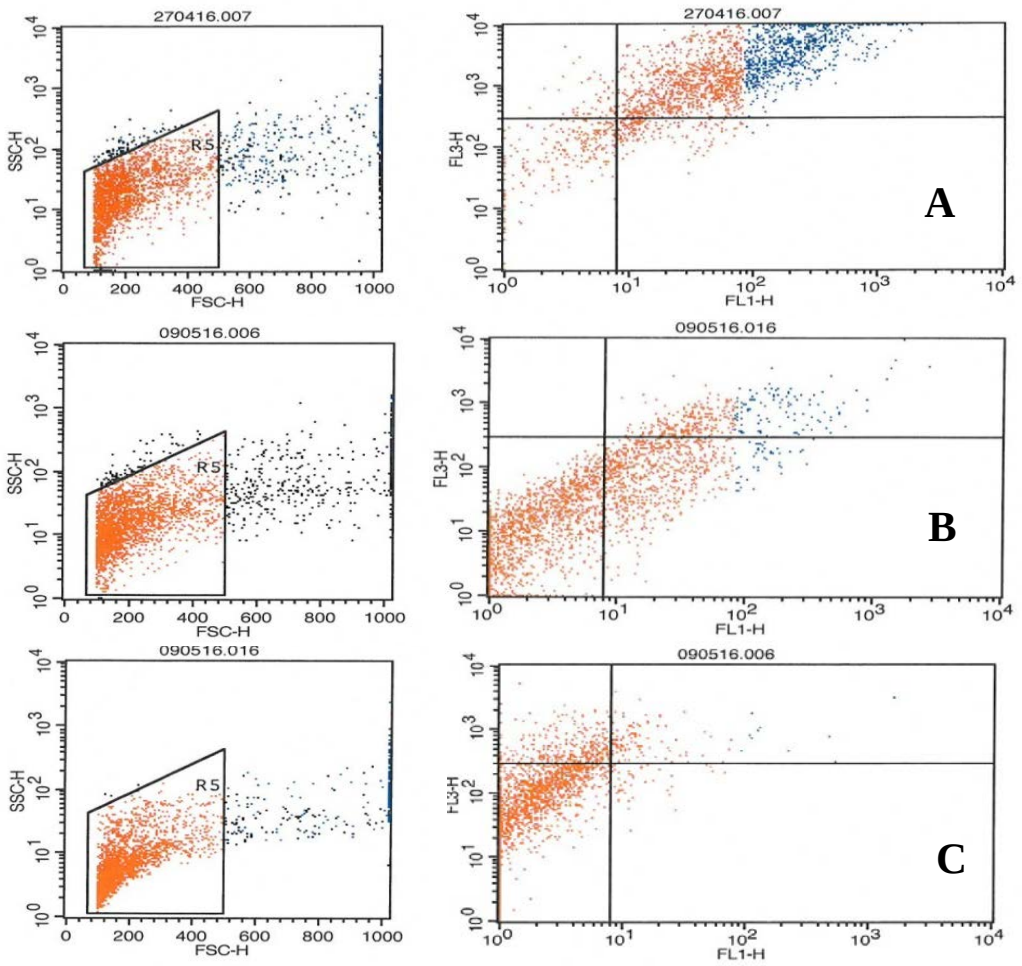

Figure 3. Flow cytometry results for *E. coli* after treatment with Pentane (1 M) (A) ,*S. oneidensis* after treatment with $ZnCl_2$ (1 M) (B) and *M. trichosporium* (OB3b) after treatment with DDVP (1 M) (C) , respectively.

Table 2. Flow cytometry data showing the percentage of live and dead bacteria before and after treatment with different pollutants.

Type of bacteria	Type of pollutant	Before		After	
		Live	Dead	Live	Dead
E. coli	$ZnCl_2$	73.88%	26.12%	26.11%	73.89%
S. oneidensis	$ZnCl_2$	52.32%	37.68%	67.68%	32.32%
M. trichosporium (OB3b)	$ZnCl_2$	71.49%	28.51%	24.49%	75.51%
E. coli	DDVP	85.13%	14.87%	29.43%	70.57%
S. oneidensis	DDVP	71.32%	28.68%	66.71%	33.29%
M. trichosporium (OB3b)	DDVP	85.33%	14.67%	32.33%	67.67%
E. coli	Pentane	86.54%	13.46%	31.54%	68.46%
S. oneidensis	Pentane	77.71%	22.29%	33.68%	66.32%
M. trichosporium (OB3b)	Pentane	79.47%	20.53%	62.58%	37.42%

Direct evidence of cell enlargement was obtained from SEM study. SEM images (Figure 4) show the rupture of *E. coli* and *M. trichosporium* (OB3b) bacteria (Fig. 4A and 4C) and enlargement of *S. oneidensis* cells (Fig. 4B) caused by exposure to high concentration (1 mol) of $ZnCl_2$. These observations are similar to previously reported SEM studies of bacteria.[37,38] In contrast, *S. oneidensis* bacteria were affected much less by $ZnCl_2$ than the other bacterial strains and appeared slightly elongated (Figure 4B). Similar elongation has been observed in *S. aureus* due to exposure to high salt concentration as a specific response to other stress conditions.[39] Also, a significant increase in bacteria length was found in *S. oneidensis* exposed to UV radiation.[40] The data above are consistent with the conclusion that *E. coli* cells are strongly inhibited by all three pollutants, while *S. oneidensis* are less affected by Zn^{2+} ions as compared to the strong inhibition effect of DDVP and pentane. *M. trichosporium* (OB3b) cells are severely affected by Zn^{2+} ions and DDVP, while pentane/ethanol mixture may even stimulate their growth.

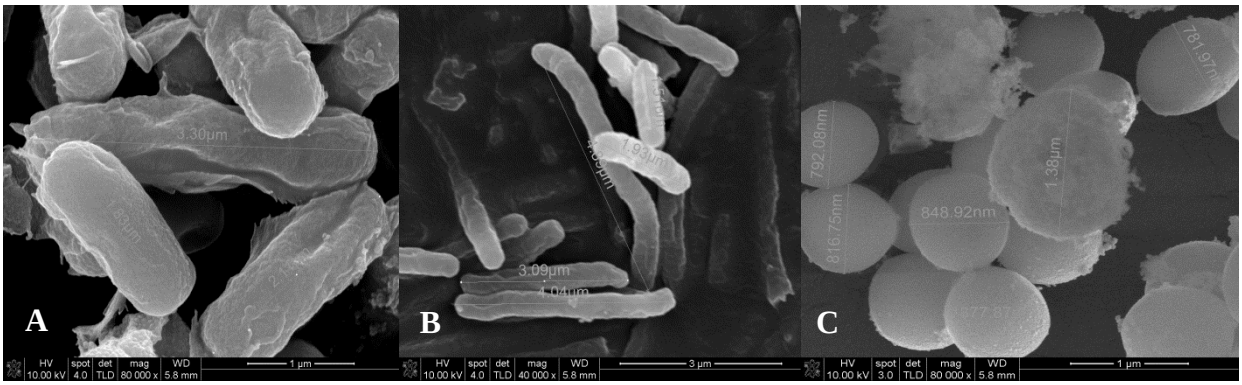

Figure 4. SEM images of (A) *E. coli* after treatment with pentane (1 M), (B) *S. oneidensis* after treatment with $ZnCl_2$ (1 M) and (C) *M. trichosporium* (OB3b) after treatment with DDVP (1 M).

Among the three optical methods used to determine the proportion of live and dead bacteria, flow cytometry appeared to be the most reliable.[41] Flow cytometry is also expected not to be affected by different motility of *E. coli*, *S. oneidensis*,[26] and *M. trichosporium* (OB3b). Immotile dead bacteria may sediment more readily, which may affect the results of static fluorescent microscopy and optical density measurements.

Electrochemical study of bacteria in suspensions and immobilized bacteria

The effect of Zn^{2+} ions, DDVP, and pentane in of all three bacterial strains, both in suspension culture and immobilized on the screen printed gold electrodes, was studied with cyclic voltammetry. A typical series of CVs recorded on *E. coli*, *S. oneidensis*, and *M. trichosporium* (OB3b) samples are shown in Figure 5. The CV curves in Figure 4 are almost featureless in the selected voltage range from -0.5 V to +0.5 V, which was chosen in order to avoid electrochemical reactions on the electrodes, with the scan range limited to where both cathodic and anodic currents just began to rise. The values of both cathodic and anodic current at -0.5 V and +0.5V, respectively, depend on the bacteria concentration in solution,[25,26] however the effect on anodic current is more pronounced and it was therefore used for analysis in this work.

CV cycles shift progressively upwards upon increasing the pollutants concentration from zero to 1 mol (Figure 5). The characteristic parameter in this study, e.g. the value of anodic current at +0.5 V, increases with the increase in pollutant concentration for all three bacteria in both liquid and immobilized forms. This means that the electrical conductivity is controlled by bacteria adsorbed on the surface of gold electrodes and acting as insulating layer reducing the current. The correlation between bacteria cell density and the electric current (or conductivity) values is very important for further study of the effect of pollutants, and such measurements were always carried out first.[25,26] The presence of pollutants (Zn^{2+} ions, DDVP, and pentane in our case) causes the damage of bacterial cells, and therefore bacteria became less insulating, in-turn leading to the increase in the anodic current, which is observed in Figure 5.

To analyse the effect of pollutants on electrical properties of immobilized bacteria, the values of anodic current (I_A) at +0.5V from CV measurements were normalised by the currents values of uncoated electrodes in PBS with the addition of a particular pollution of particular concentrations (I_{A0}) to construct the values of relative changes of anodic current. For example, for *S. oneidensis* bacteria treated with 1 mM solution of pentane (Figure 5A), the reference was recorded on uncoated electrodes in PBS containing 1mM of pentane.

The relative changes in anodic current are presented in Figure 6 for all three bacteria studied as concentration dependences of the three pollutants. As one can see the effects of $ZnCl_2$, DDVP, and pentane on *S. oneidensis,M. trichosporium* (OB3b) strain and *E. coli* are distinctly different. *E. coli* appeared to be affected by $ZnCl_2$, DDVP, and pentane even at low concentrations since the $\Delta I_A/I_{A0}$ values increase monotonically in Figure 6A, 6B, and 6C, respectively. This means that *E. coli* is equally inhibited by all three pollutants and becoming less electrically resisting. In contrast, *S. oneidensis* is almost unaffected by $ZnCl_2$ at low concentrations of all pollutants up to 10 mM, and then $\Delta I_A/I_{A0}$ started to increase at high concentrations of 100mM and 1M. Such behaviour of immobilized *E. coli* and *S. oneidensis* bacteria is similar to those in liquid as reported in.[26.]

M. trichosporium (OB3b) responded to $ZnCl_2$ (Figure 6A) and DDVP (Figure 6B) similarly to the other two bacteria studied,though the changes in $\Delta I_A/I_{A0}$ are more pronounced at high pollutant concentrations, particularly for pentane. However, *M. trichosporium* (OB3b) is not affected by pentane (Figure 6C) even at high concentration; moreover an overall trend of small decrease in $\Delta I_A/I_{A0}$ is observed. Such behaviour was expected since methanotrophic bacteria can oxidise many hydrocarbons.[29]

The results presented in Figure 6 show a possibility of pattern recognition the three pollutants studied. The relative responses of the three bacteria (*E.coli*, *M. trichosporium*, and *Shewanella oneidensis*) to the three pollutants ($ZnCl_2$, DDVP, and pentane) presented in a pseudo-3D plot in Figure 7, clearly demonstrated this. The experimental points for $ZnCl_2$, DDVP, and pentane in concentrations up to 1M shown in different colours are well-separated in the 3D-graph in Figure 7. This is a clear indication that pattern recognition principles can be applied for identification of pollutants using different types of bacteria.

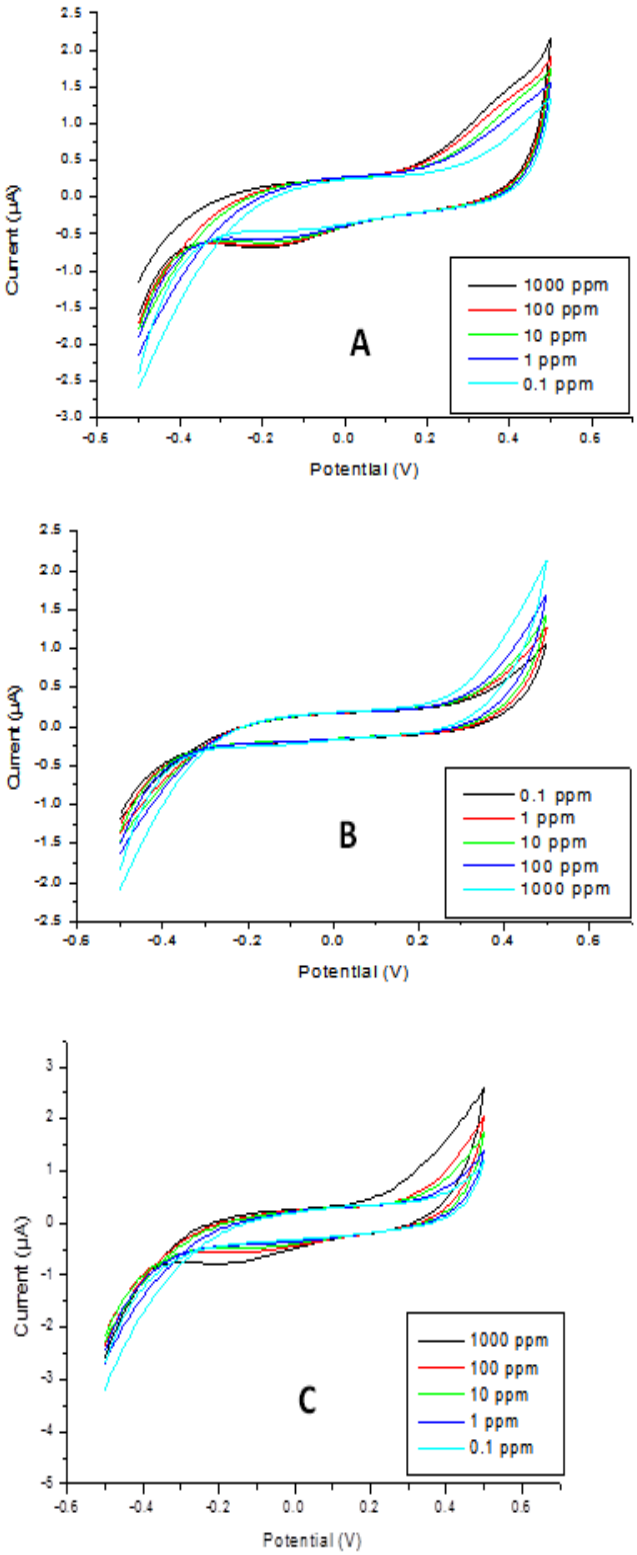

Figure 5. Cyclic voltammogram recorded (A) on immobilized *S. oneidensis* treated with different concentration of pentane, (B) *E. coli* treated with different concentration of DDVP and (C) *M. trichosporium* (OB3b) treated with different concentration of $ZnCl_2$; CV curves for clear LB broth are shown on all graphs.

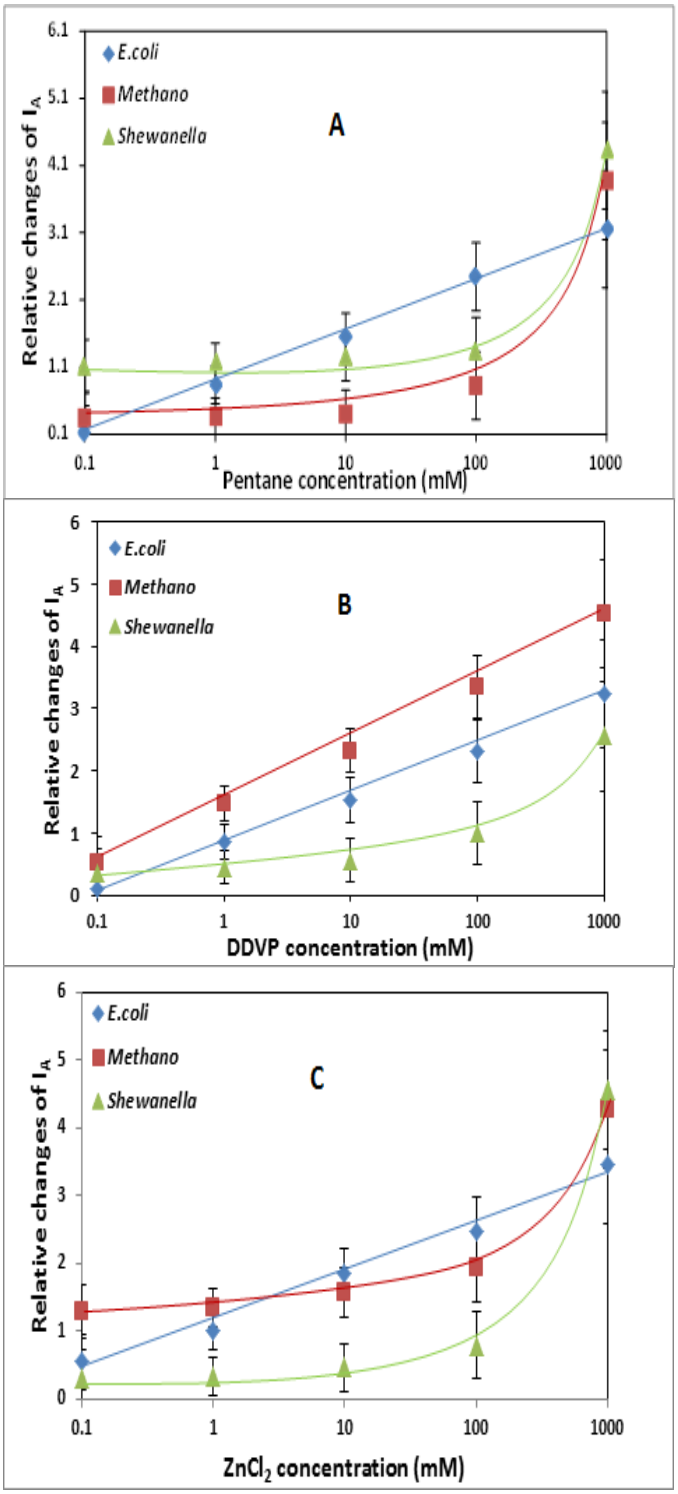

Figure 6. Comparison of relative changes of anodic current (IA) at +0.5V of all three types immobilized bacteria samples on modified electrodes exposure to: (A) pentane, (B) DDVP and (C) $ZnCl_2$.

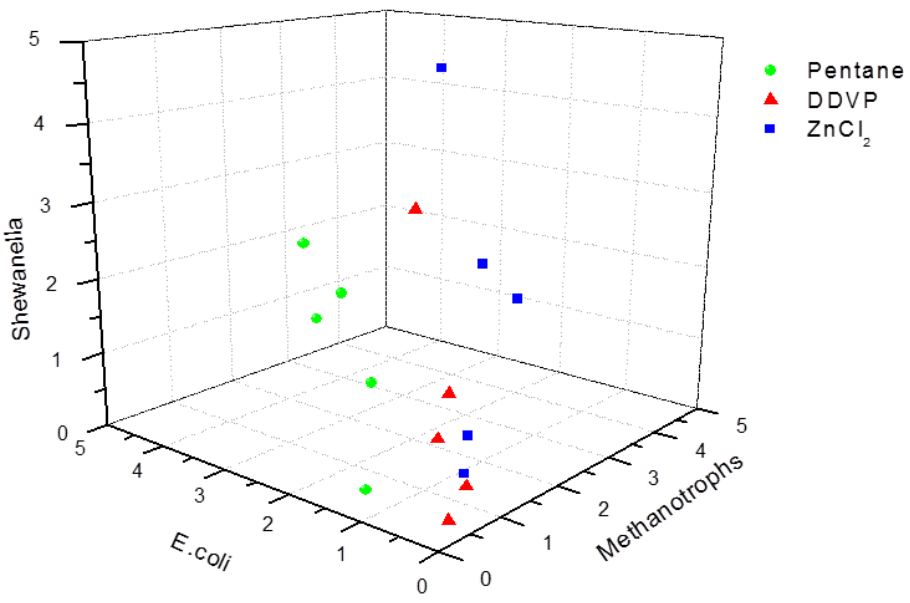

Figure 7. 3D plot of relative changes in anodic current for *E. coli*, *M. trichosporium* (OB3b) and *S. oneidensis* caused by different pollutants. Points show the direction of the pollutants' concentration increase from 0.1mM to 1000mM.

The concentration of pollutants could be evaluated to using the appropriate calibration and data extrapolation.

Conclusions and future work

This study gives proof-of-principle for the use of a panel of diverse wild-type bacteria to detect and discriminate different pollutant molecules. First of all, the values of anode (or cathode) current were found to correlate with bacteria concentration and thus with the concentration of different pollutants acting as inhibitors for bacteria. It shows simple electrochemical tests, i.e. cyclic voltammograms, either on gold electrodes immersed into liquid bacteria samples or (even better) on screen printed gold electrodes with immobilized bacteria have distinctive characteristic responses to different pollutants, and the pattern recognition principles can be applied for identification of pollutants.

This work paves the way for the development of novel, simple, and cost effective electrochemical bacteria-based

sensor array for preliminary assessment of the presence of pollutants in water. Future work which is currently underway will focus on extending the range of pollutants (different heavy metals, pesticides, and petrochemicals) and using advanced data processing tools such as (ANN) Artificial Neuron Network for analysis of real water samples.

Acknowledgements

The authors would like to thank the Iraqi Government, Ministry of Higher Education and Scientific Research and University of Basrah for sponsoring the PhD project.

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Received: 09.12.2018.
Accepted: 07.02.2019.