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Characterization of thermostable aminoacylase from *Geobacillus* sp. strain SZN

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**Abstract.** Aminoacylase (EC 3.5.1.14) hydrolyzes N–acetylated amino acids to produce amino acids. Although thermostable aminoacylase has been commercially produced since 2004, there was a knowledge gap in the field of understanding aminoacylase thermostability from a structural point of view. This study investigated the physical and structural properties of the purified thermostable aminoacylase SZN. The spectropolarimetry data for structural determination has indicated a gradual decrease of α-helix from 36 to 27.6%, followed by tremendous disorientation of the structure at the transition of temperatures from 60 to 70°C (27.6 to 19.5%). In contrast, the percentage of β-sheet has increased steadily over the tested temperatures. The α-helix, where notable metal binding and catalytic residues are located, was totally weakened at temperatures above 70°C, thus resulted in loss of activity. The loss of the α-helical structure could further explain drastic deterioration of activity at temperatures beyond 70°C. The activity of aminoacylase SZN was enhanced by divalent metal ions, such as Mn$^{2+}$ and Cu$^{2+}$, and inhibited by detergent Triton-X-100. As a conclusion, the isolated aminoacylase SZN was characterized as a thermostable enzyme based on the α-helical structure integrity and functional stability in high temperatures. This enzyme could be used as an alternative enzyme for bioindustries in view of its activity enhancement in high temperatures and stability in various tested inhibitors.

**Keywords:** aminoacylase; *Geobacillus* sp.; secondary structure; thermostable enzyme; α-helix

**INTRODUCTION**

Global amino acid market demand was 7.5 million tons in 2016 and was expected to reach a volume of more than 10 million tons in 2020 (Research & Market, 2015). The market of amino acids is anticipated to reach USD 30.8 billion by the end of 2024. This can be attributed to various factors such as growing consumer awareness and the rising demand for healthy and functional foods. The search for new local thermostable aminoacylase is needed in the global industry, especially in the industry that focusing on productions of amino acids. Enzymes from extremophiles are more attractive compared to other common enzymes because they can perform industrial processes even under harsh conditions, under which common proteins are completely denatured. In common cells, enzymes will be denatured and unfolded when the
temperature increased beyond a specific point. The rate of molecular movement and reaction increased when the temperature increased, but at the same time, there is a progressive inactivation caused by denaturation of the enzyme protein (Robinson, 2015). This is because the three-dimensional structures of proteins are damaged resulted from breakage of hydrogen and disulphide bonds that are responsible to maintain the enzyme structure. Protein unfolding may cause the enzyme unable to catalyze the reactions appropriately because it had lost its fundamental shape which is associated with its specific affinity and its substrate binding site. This work was emphasized on studying thermostable aminoacylase SZN secondary structure stability and its distortion at high temperatures. Furthermore, providing a better understanding of the adaptation of thermostable aminoacylase at high temperatures, from the structural point of view is our subsequent objective.

**MATERIALS AND METHODS**

**Source of bacterium**
The water samples (59°C) were collected from Ulu Slim Hot spring in Perak (GPS coordinate: 3.8988° N, 101.4979° E). A thermophile from *Geobacillus* species, identified as a new strain known as strain SZN, was isolated (Adenan et al., 2018), and the aminoacylase extracted from the bacteria cells was applied throughout this work to study its biochemical properties and characteristics.

**Aminoacylase assay**
Aminoacylase activity was determined in 0.5 mL assay mixtures containing 0.2 mL of aminoacylase enzyme in 50 mM Tris-HCl (pH7.5) and 0.3 mL of 10 mM of N-acetyl-L-methionine (NAMET) substrate. The 0.5 mL assay mixtures were shaken for 30 min with shaking at 200 rpm, 60°C. The reaction was stopped using 0.5 mL of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation method with 4000 rpm for 1 min. Then, 1 mL supernatant was mixed with 0.25 mL of 3% ninhydrin solution and 0.25 mL of 250 mM acetic-cyanide (pH5.0) buffer. The mixture was boiled for 15 min and cooled to ambient temperature by the addition of 1.5 mL of 50% (v/v) isopropanol. The liberated L-methionine was measured using Biomate 3S UV visible spectrophotometer (Thermo Fisher Scientific, USA) at absorbance wavelength of 570 nm (A_{570}). L-methionine amount was determined from standard curve that was prepared according to Moore and Stein (1948).

**Preparation of cell extract**
The crude cell extract for the assay was prepared from 1 L of the culture growth at 60°C overnight in LB broth. The supernatant of the culture was removed by centrifugation at 12,000 rpm and 4°C for 10 min. The precipitated cells were washed with 50 mM Tris-HCl (pH7.5). The cells were then resuspended in 50 mL of 50 mM Tris-HCl (pH7.5) and disrupted by using ultrasonic disruptor UD-200 at 60 W for 4 min intermittently in an ice bath. Cell extract was obtained by centrifugation at 12,000 rpm for 10 min at 4°C. The cell extract was subjected to aminoacylase assay at a different range of temperatures (30, 40, 50, 60, 70, 80, and 90°C) for characterization, and at 60°C for standard assay.

**Aminoacylase purification steps**

**Ion exchange chromatography (IEX)**
A flow rate of 0.7 mL/min was used during crude enzyme sample injection into XK16 column containing 10 mL of DEAE-Sepharose Fast Flow resin (GE Healthcare, Sweden). Prior to sample injection, the resin was pre-equilibrated with 50 mM Tris-HCl (pH 7.5), as the binding buffer. The column was then washed with 10 column volumes (cv) of the binding buffer followed by ascending linear gradient elution with 25 cv of NaCl varying from 0 to 1.0 M. Eluate size was set at 2.0 mL per fraction, in which all fractions were subjected to aminoacylase assay and dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to observe the protein size and enzyme purity. Fractions with high enzyme activity and less contaminants (unwanted proteins) were pooled and subjected to gel filtration purification step by using Sephacryl (S-200) HR resin (GE Healthcare, Sweden).

**Gel filtration chromatography**
The fractions from IEX with high aminoacylase activity were pooled and concentrated up to 0.8%
of the total column volume (80 mL resin) by using molecular weight cut-off spin column (10.0 kDa) (Milipore, USA) at 10,000 rpm, 4°C. The concentrated sample was then loaded into S-200 column with flow rate 0.2 mL/min using the peristaltic pump. The sample was eluted using 50 mM Tris-HCl (pH 7.5) buffer. Eluate size was set at 1.0 mL per fraction, in which all fractions were subjected to aminoacylase assay and SDS-PAGE to observe the protein size and purity. The protein content was determined by the Bradford (1976) method using the Bio-Rad assay reagent and Bovine serum albumin as the standard. The protein concentration from each step of purification was monitored by measuring their absorbance at 280 nm.

**Thermal characterization of aminoacylase SZN**

**Assessment of aminoacylase secondary structure stability by circular dichroism (cd)**

Mechanisms of protein stability in various temperatures were analyzed using JASCO J-810 Circular Dichroism Spectropolarimeter (Tokyo, Japan). By utilizing several parameters pre-programmed in Spectra Manager software (JASCO, Japan), the generated CD spectra can be used for analysis in secondary structure changes as well as thermal denaturation. Secondary structures estimation was determined by the Bradford (1976) method using the Bio-Rad assay reagent and Bovine serum albumin as the standard. The protein concentration from each step of purification was monitored by measuring their absorbance at 280 nm.

**Thermal denaturation analysis**

The measurement of thermal denaturation ($T_m$) was acquired from ellipticity at 222 nm of a completely closed 10.0 mm cell containing 1.0 mg/mL native enzyme solutions, which was heated at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100°C at 1°C/min heating rate. Data pitch, bandwidth, response, and scanning speed were programmed as 0.1 degree, 1 nm, 8 seconds and 1 degree per minute.

**Effect of temperature on aminoacylase activity**

Investigation on hydrolysis of N-acetyl-L-Methionine (NAMET) by aminoacylase SZN at temperatures 30, 40, 50, 60, 70, 80, and 90°C was conducted at a shaking rate of 200 rpm for 30 min. Aminoacylase activities that exhibited in various temperatures were compared in relative to activity at 60°C that served as control (100%).

**Effect of temperature on enzyme stability**

Pre-incubation of the enzyme in temperatures of 5, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C for 30 min in 200 rpm water bath shaker was carried out prior to aminoacylase assay, in order to determine the enzyme temperature stability. Activity at 5°C was selected as a standard (100%).

**Effect of exposure time at 60°C on aminoacylase SZN**

The half-life of the enzyme was examined by pre-incubating the protein at 60°C from 0-36 hours in a water bath shaker. Samples were removed at intervals of 4 h for measurement of activity, in which the activity at 0 min is a control (100%) of the experiment.

**Other characterization of thermostable aminoacylase SZN**

**Effect of pH on aminoacylase activity and stability**

The optimum pH of aminoacylase SZN activity was determined by the enzymatic hydrolysis of NAMET in 50 mM of different buffers. The buffering systems used were sodium acetate (pH 4, pH 5, pH 6), potassium phosphate (pH 6, pH 7, pH 8), Tris-Cl (pH 8, pH 9), sodium tetraborate (pH 9, pH 10, pH 11), and sodium hydrogen phosphate (pH 11, pH 12). The substrate was resuspended with buffers at various pHs with ratio 1:3 (v/v). For pH stability test, aminoacylase was pre-incubated with buffers of tested pHs at ratio of 1:3 (v/v) at 60°C and 200 rpm for 30 min followed by enzyme assay. Enzyme activity in 50 mM phosphate buffer (pH 8.0) was further chosen as control for this experiment.

**Effect of metal ions on aminoacylase activity**

In this study, the enzyme was pre-treated with 0, 1.0 and 10.0 mM of chloride (Cl) metal ions, such as Na+, K+, Mg2+, Ca2+, Mn2+, Co2+, Ni2+, Cu2+ and Zn2+ for 30 min at 60°C before subjected to enzyme assay.
Effect of inhibitors on aminoacylase activity
Denaturing and reducing agents, such as β-mercaptoethanol, Triton-X-100, Tween 20, sodium dodecyl sulphate (SDS) and dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), were used at final concentrations of 5.0 and 10.0 mM to treat the protein at 60°C for 30 min. Inhibitor-free mixture was considered to be the negative control in the colorimetric assay.

RESULTS

Purification of aminoacylase from Geobacillus sp. strain SZN
Aminoacylase SZN was successfully purified using Ion Exchange DEAE Sepharose Fast Flow chromatography (IEX) (Figure 1) and Sephacryl S-200 Gel Filtration chromatography (GF) (Figure 2). The purification results are summarized in Table 1. The enzyme purity was obtained at 14.04 fold, compared to crude, with final yield at 16.53% utilizing only two steps of chromatography, caused its specific activity of the purified enzyme increased to 66.4 U/mg at the final step compared to specific activity from crude which was 4.73 U/mg. The protein sample from each purification step was analyzed by SDS-PAGE and the purity of aminoacylase SZN from each purification step can be observed in Figure 3, shows that the purified protein was resolved almost as single band at ~42 kDa.

Characterization of aminoacylase strain SZN
Assessment of aminoacylase secondary structure stability by CD
The protein stability in various temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C) was analyzed using JASCO J-810 CD Spectropolarimeter (Figure 4) and estimated the percentage of α-helix and β-sheet secondary structures by using Perry Freeshell server (Raussens et al., 2003) (Table 2). The results showed that the α-helix was still maintained its original structure even heated up to 60°C. At 10-40°C, the percentages of α-helix and β-sheet were maintained in a range of 34-37% and 16-19%, respectively. The α-helix was appeared at 28-32% and β-sheet at 19-23% when temperatures increased to 50-60°C. At 100°C, aminoacylase SZN significantly lost its α-helical conformation and changed to β-sheet as each of the conformation was recorded at 12% and 33%, respectively.

![Figure 1](image-url)  
*Figure 1.* Ion-Exchange Column Chromatography of cell extracts from *Geobacillus* strain SZN by using DEAE Sepharose Fast Flow column. Each step in the protein purification protocol: FT, flowthrough; W, washing unbound; E, elution and WAE, wash after elution, is separated by dashed lines. The gradient blue dashed line indicates the gradient concentration of NaCl in elution steps. Absorbance at 280 nm and aminoacylase activity was indicated by (■) and (▲), respectively.
Table 1. Purification table of aminoacylase from *Geobacillus* strain SZN by Ion Exchange and Gel Filtration S-200 purification steps.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein content (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Enzyme purity (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>2130</td>
<td>450</td>
<td>4.73</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>IEX*</td>
<td>635</td>
<td>32.5</td>
<td>19.5</td>
<td>4.12</td>
<td>29.8</td>
</tr>
<tr>
<td>S-200+</td>
<td>352</td>
<td>5.3</td>
<td>66.4</td>
<td>14.04</td>
<td>16.53</td>
</tr>
</tbody>
</table>

*Ion Exchange Chromatography*  
*Gel Filtration Chromatography*

Figure 2. Sephacryl S-200 Gel Filtration Chromatography of pooled fractions of protein from IEX. Absorbance at 280 nm and aminoacylase activity was indicated by (▲) and (■), respectively.

**Thermal denaturation analysis**

Melting point analysis is an assessment of the dissociation-characteristics of protein secondary structure during heating. As the temperature raised, the helical structures begin to dissociate, leading to a rise in the absorbance intensity at 222 nm. The temperature at which 50% of protein denatured is known as the melting point. The result was found to be parallel with its aminoacylase activity optimal temperature, where the enzyme started to lose 50% of the activity at above 80°C (Figure 5).

**Effect of temperatures on aminoacylase activity and stability**

The aminoacylase SZN relative activity was increased gradually from 37.6% to 100% at the temperatures from 30-60°C and decreased from 66% to 27% when increased temperatures from 70 to 90°C. The aminoacylase SZN optimum temperature was detected at 60°C (Supplementary Material 1a). Stability of aminoacylase SZN from 5°C to 70°C was supported by its relative aminoacylase activity in Supplementary Material 1b in which a significant lost (more than 50%) was observed when the temperature increased to 80°C and above. At 70°C, the stability of aminoacylase SZN was 73% (Supplementary Material 1b).

**Effect of exposure times at 60°C on aminoacylase SZN**

An in-depth investigation on the half-life of aminoacylase SZN was carried out at 60°C. The half-life for the enzyme was deduced at 16 h (Supplementary Material 1c). Relative activity of aminoacylase SZN was reduced from 47% to 28% when incubated at 20, 24, 28 and 32 h.
Table 2. Estimated percentage of secondary structures by using Perry Freeshell Software when protein was incubated at various temperatures.

<table>
<thead>
<tr>
<th>Secondary structure (%)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>α-helix</td>
<td>36.0</td>
</tr>
<tr>
<td>β-sheet</td>
<td>17.2</td>
</tr>
<tr>
<td>Random</td>
<td>31.0</td>
</tr>
</tbody>
</table>

Figure 3. SDS-PAGE of aminoacylase SZN purification. Line 1, 2, 3, and 4 at top of the gel are samples from Unstained Protein Molecular Weight Marker (Fermentas, Canada), crude enzymes, IEX, and gel filtration, respectively. Components with their respective sizes (kDa), found in standard protein marker are: β-galactosidase, 116.0; bovine serum albumin, 66.2; ovalbumin, 45.0; lactate dehydrogenase, 35.0; REase Bsp98I, 25.0; β-lactoglobulin, 18.4 and lysozyme, 14.4.

Figure 4. Far UV spectra of aminoacylase at various incubation temperatures.

Figure 5. CD spectra for determination of thermal denaturation point of aminoacylase. A curve that indicates the transition state of the α-helical structure was formed when the temperature raised from 70-80°C. The temperature of 80°C at approximate midpoint of the sigmoidal curve has defined as thermal denaturation point of aminoacylase.

Other characterization of thermostable aminoacylase SZN

Effect of pH on aminoacylase activity and stability
In Supplementary Material 2a, the relative activity appeared low at pH 4 (22.4%) and accelerated to optimum activity (100%) when pH increased to 8. However, the relative activity was significantly dropped after pH 9 where the relative activity lost almost 80% at pH 12, indicating that aminoacylase SZN works more efficiently at neutral pH environment. To test its stability in broad range of pH, aminoacylase SZN was added to different buffers with different pHs. At pH 4, the relative activity of the enzyme was 59%. As the pH increased from 5 to 9, the relative activity was restored to 81% at pH 9 and 100% at pH 8. Further increase of pH 10 and pH 11 had exhibited declination of the activity until 46% and totally lost at pH 12.

Effect of metal ions on aminoacylase activity
Aminoacylase is a metalloenzyme, from M20 family that contained with at least one zinc ion for
each subunit enzyme assisting in catalytic process. Treatment of aminoacylase SZN with 1 mM and 10 mM Zn$^{2+}$ did inhibit the activity, but strongly enhanced the thermostability, suggesting that additional Zn$^{2+}$ may occur at the second metal binding site and induce a possible alteration in enzyme conformation to improve thermostability (Tanimoto et al., 2008). Our results showed that 1 mM metal ion reduced the aminoacylase activity, whereas the 10 mM Cu$^{2+}$, Mg$^{2+}$, Mn$^{2+}$ and Na$^{+}$ increased the aminoacylase activity to 107, 153, 160 and 136%, respectively (Supplementary Material 2c). We postulate that increased concentration of divalent ions, such as Mg$^{2+}$ and Mn$^{2+}$, may result in enhancement of activity, probably due to increased stability in enzyme-substrate and product complexes (Knap et al., 2017; Rahman et al., 2011).

**Effect of reducing and denaturing agents on aminoacylase activity**

Tween 20 and Triton X-100 demonstrated inhibition effects at concentrations of 5 and 10 mM, where the relative activity was decreased from 90.7 to 85.8% and 73 to 66.4%, respectively. At 10 mM, inhibition effects occurred in enzyme assay containing PMSF and SDS with its relative activity decreased to 91.6% and 85.4%, respectively. Treatment of aminoacylase SZN with EDTA at 5 and 10 mM did not inhibit the activity due to weak affinity of EDTA to Zn$^{2+}$ (Tanimoto et al., 2008). This observation was supported by Sakanyan et al. (1993) who reported that the activity of aminoacylase from *B. stearothermophilus* can only be diminished at 50 mM EDTA.

**DISCUSSION**

Aminoacylase enzyme is classified into the M20A family of metallopetidases, in which zinc is needed as an essential metal for catalytic activity (Lindner et al., 2003). Based on our reported data regarding amino acid sequence alignment of aminoacylase SZN with *Pyrococcus horikoshii* and several other species, the conserved regions of metal binding residues (Glu139 and Glu140), and catalytic residue (Arg261), were located at $\alpha$-helical structure (Adenan et al., 2018). Similarly, Tanimoto and colleagues (2008) also revealed that the positions of metal binding site residues for *Pyrococcus horikoshii* were found at His106, Glu139, Glu140 and His164 and catalytic residues at H198 and A260. These findings are essential to understand the aminoacylase thermostability at structural point of view, as significant loss of $\alpha$-helical structure was observed at temperatures beyond 70°C, and thus, preventing the metal binding and catalytic residues to be folded into its catalytically active conformation. Moreover, the melting point of aminoacylase SZN was recorded at 80°C (Figure 5), which was in agreement with the observation obtained from Supplementary Material 1a.

Thermostability of aminoacylase SZN may be assisted by existence of metal ion as ligand during catalysis reaction. This was supported by finding of $\alpha$-helix at the predicted catalytic and metal binding sites which is known to contribute to high portion of hydrogen bonds. More than 50% $\alpha$-helix structure was predicted by using SOPMA (Deleage & Geourjon, 1995) tool in Expasy for computation of secondary structures $\alpha$-helix, $\beta$-strand, and random coils, that is inferred to play a crucial role in maintaining the active conformation (Adenan et al., 2018). Native and active proteins are held together by a delicate balance of non-covalent forces among the secondary structures (hydrogen bonds, ion pairs, and hydrophobic and Van der Waals interactions). When high temperatures disrupt these non-covalent interactions, proteins become unfold and inactive.

The inhibitory effect of divalent ions, such as Zn$^{2+}$ and Ni$^{2+}$, was clearly observed on aminoacylase SZN. These ions have been reported to inhibit the proteolytic activities of elastase strain K (Rahman et al., 2011), ME-4 (Cheng et al., 2009) and PseA (Gupta et al., 2005) which also happened to be metalloproteases. The highest half-life of aminoacylase was reported by Hollingsworth (2002), who found that the stability of aminoacylase from *Thermococcus litoralis* was 25 h at 70°C. Aminoacylase SZN can withstand a broad range of inhibitors and did not exhibit significant effects on its stability in detergent especially Tween 20. Thus, the aminoacylase SZN can be potentially applied in industries, including pharmaceutical and food and beverage industries. Similar characteristics were reported for protease...
from Bacillus horikoshii, whereby the enzyme has shown excellent potential as an additive in feed and detergent industries due to its stability in SDS, oxidizing agents and organic solvents (Joo & Choi, 2012). Characterization on aminoacylase SZN activity and stability in different pHs has shown that the enzyme optimum activity is ranging from pH 6-8, similar to those optimum pHs for P. furiosus, Streptomyces mobaraensis and Deinococcus Radiodurans R1 at pH 6.5 (Story et al., 2001), pH 7.5 (Koreishi et al., 2009) and pH 8 (Lin et al., 2007), respectively.

**CONCLUSION**

The wild-type aminoacylase SZN was purified in two steps, namely ion exchange and gel filtration chromatography, with approximately 14-fold of specific activity compared to crude sample (Table 1). From this work, the structural properties of thermostable aminoacylase from Geobacillus sp. have unveiled the protein folding and unfolding mechanism involving α-helical structure in high temperatures. Thus, the findings reported in this communication provide insights into characteristics, adaptation mechanism and behaviour of enzymes in various temperatures, especially at the estimated α-helical structure of aminoacylase SZN active site, which is known to assist the enzyme stability at high temperatures.

**ACKNOWLEDGEMENTS**

We would like to thank The Ministry of Higher Education, Malaysia (RACE 2012-0145-102-62) for their financial aid in this project. Besides, we would like to dedicate our special appreciation to Malaysia Genome Institute for facilitating CD analysis.

**REFERENCES**


Effect of fungal filtrates on germination of wheat grains and the biological control of these fungi using black pepper extract

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Abstract. Wheat is one of the world's most abundant and essential food crops. It covers a significant area of the earth's surface, higher than any other plant, and tends to be among the top strategic crops. Wheat contamination with fungi leads to rapid deterioration of quantity and quality of wheat products. Many of those fungi are potential mycotoxins producers. This study aimed to isolate and identify fungi that contaminating the wheat grains from the Misurata Agricultural Research Center area and the South Region of Libya. Fungi contaminating wheat grains were isolated on Potato Dextrose Agar (PDA) and identified by culture characteristics and microscopically. Fungal filtrates of two fungal isolates, Aspergillus niger and Rhizopus sp., were tested for their effects on the germination and seedlings of wheat grains. Furthermore, the effect of acetic extracts of Black pepper (Piper nigrum) on the growth of the isolated fungi was also investigated. Ten types of fungi belonging to four genera were isolated and identified. The germination rate of wheat grains irrigated with the filtrate of A. niger and Rhizopus sp. was 20% and 80% respectively, compared with 100% of the control grains, which were irrigated with water. The culture filtrates of both A. niger and Rhizopus sp. affect not the only percentage of grains germination but also the morphology of wheat seedlings. It adversely affected the length of the radicles and coleoptiles. The acetone extract of P. nigrum showed inhibitory effect (85.7% ± 3.7 and 44.0% ± 3.1) on the germination of A. niger and Rhizopus sp. respectively. This study concludes that fungal secretions have pathogenic effects on plant growth, which can lead to potential health risks for the human population. Biological control such as Piper nigrum extracts can be an alternative to chemical pesticides for controlling fungal pathogens and their secretions.

Keywords: wheat grains, Aspergillus, Rhizopus, Piper nigrum, germination, biological control

INTRODUCTION

Wheat is one of the most important food crops in the world. It contains carbohydrate (70%), protein (12%) and fat (29%), providing about 20% of the calories in human food worldwide (Putnam et al., 2002). Wheat grains are exposed to various fungal contaminations starting from the time of physiological maturity until they are used for either agriculture or consumption. More than 100 fungal species have been found contaminating the grains. Some of these fungi
have potential risks to plants grown from these grains, while others can cause problems of decay and release of toxins during storage that has a potential threat to the consumers’ health (Kabak et al., 2006; Kent & Evers, 1994). The safety of grains is essential for its use in agriculture, storage, and consumption. Hence, wheat grains should be stored in a proper condition which contains suitable moisture (not exceed 12%), absent of contaminants, residues or harmful substances, grain fracture percentage is not more than 2%, and should be of the same size, color, and smell. The storage conditions should be as appropriate as possible to maintain good grain quality (Lopez-Garcia & Park, 1998).

Wheat grains show the symptoms of deformation, discoloration, or black spots caused by some fungi in the field when the humidity is high (>90%). The most common fungi associated with these symptoms include Alternaria sp., Fusarium sp., Cladosporium sp., Aspergillus sp., Penicillium sp., and Rhizopus sp. (Bhale et al., 2001). Fungal contamination of soybean seeds with Aspergillus, Penicillium, and Rhizopus has shown to cause reduction of germination rate and defected of seedling during field plantation (Anwar, 1995; Ibrahim, 2015).

Black pepper (Piper nigrum) is one of the well-known medicinal plants that have prominent roles in pharmaceutical, industrial and agricultural production. It contains active antimicrobial substances, aromatic oils, phenols, aldehydes, and alkaloids. These substances are used in food maintenance, formulation of pharmaceuticals and alternatives to medicines and natural remedies (Jafeer & Kheirallah, 2017; Ody, 2017). P. nigrum is a flowering vine of the Piperaceae. It is cultivated for its fruit which is usually dried and used as a spice and seasoning that are known as a peppercorn. When fresh and fully mature, it is dark red and contains a single seed. Peppercorns and the ground pepper derived from them may be described just like pepper, or more precisely as black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit), and white pepper (ripe fruit seeds). Black pepper is native to Kerala in Southwestern India (Hajeski, 2016; Sen, 2004) and is extensively cultivated there and elsewhere in tropical regions. Vietnam is the world’s largest producer and exporter of pepper, producing 34% of the world’s P. nigrum crop as of 2013 (Yogesh & Mokshapathy, 2013).

Fungi of the genera Aspergillus, Fusarium, Penicillium, and Rhizoctonia are known to produce toxic metabolites which are known as mycotoxins (Eltariki et al., 2018). These mycotoxins had been reported to degrade seed quality and reduce their viability (Magan & Aldred, 2007; Mohamed-Yasseen et al., 1994). The fungistatic or fungicidal effect of spices is due to the inhibitory action of natural products, and the mechanisms involved are cytoplasm granulation, cytoplasmic membrane rupture, and inactivation and/or inhibition of intracellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition and it is also reported that plant lytic enzyme act in the fungal cell wall causing breakage of β-1,3 glycan, β-1,6 glycan, and chitin polymer (Pundir & Jain, 2010). Historically, it has been thought to cure many illnesses, such as cancer, malaria, cholera, nausea, fever, migraines, poor digestion, strep throat, and even coma. Piperine is an alkaloid found naturally in plants belonging to the pyridine group of family Piperaceae, such as P. nigrum and contains some of the antimicrobial components such as Terpinene, α-pinene, β-pinene, Linaleol and Terpineol, Piperine, pipernamide and piperamime it has to possess diverse pharmacological activities. Piperine has been found to enhance the therapeutic efficacy of many drugs, vaccines, and nutrients by increasing oral bioavailability by inhibiting various metabolizing enzymes. There is preclinical evidence that it may have modest immune system enhancing properties (Kaho et al., 2019).

Culture filtrates of Aspergillus sp. have reported in causing a reduction in seed germination and root-shoot elongation (Jalander & Gachande, 2012). Seed-borne diseases play a significant role in the quantity and quality of the agricultural product. Seed rot, seedling blight, Bipolaris leaf spot, and Cuvularia leaf spot are etiologically caused by Penicillium sp., Fusarium oxysporium, Aspergillus sp., Bipolaris maydis, and Curvularia lunata respectively (Debnath et al., 2012).

This study aimed to identify the fungal species contaminating the wheat grains in Libya and to investigate the effect of isolated fungi filtrates on
grains germination rate and seedling development. Furthermore, the inhibition effect of P. nigrum (black pepper) extract on the growth of isolated toxigenic and pathogenic fungi will also be evaluated.

**MATERIALS AND METHOD**

**Sample collection**

Wheat grain samples were collected from Misurata Agricultural Research Center area and South Region of Libya. Three samples for each type of wheat, *Triticum aestivum* cv. and the solid wheat cultivar *Triticum durum* cv., were collected randomly from stored grains in each area and transported to laboratory using properly sealed sterile polythene bags. The samples were kept at 4°C until fungal cultivation, isolation, and identification were carried out as described previously (Fente et al., 2001; Sekar et al., 2008).

**Isolation and identification of fungi on wheat grains**

Fungi carried on wheat grains were isolated on Potato Dextrose Agar (PDA) as previously described (Fente et al., 2001; Sekar et al., 2008). The wheat grains used for culture was randomly selected from the samples and picked up using sterile forceps. Grain seeds were washed aseptically with 10 mL of sterile distilled water. PDA media were prepared, and chloramphenicol (500 mg/L) was added to inhibit bacterial growth. Five grains of wheat were inoculated on each culture media plate with equal distribution. The grains were immersed in the agar to ensure that the grain surface is directly contacted with the nutrient in the medium. Three replicates were maintained for each sample. After inoculation, all dishes were incubated in the incubator at a temperature of 25°C for seven days. Culture plates were checked daily for growth and sporulation. After seven days of incubation, the different fungal colonies were sub-cultured into fresh PDA plates. The isolated fungi were identified based on colony morphology and microscopic characteristics using an optical microscope according to the following references (Domsch et al., 2007; Ebrahim, 1998; Nelson et al., 1983).

**Effect of fungal filtrates on germination of wheat grains**

Potato Dextrose Broth (PDB) was prepared under septic conditions to prepare the fungal filtrates of isolated fungi from wheat grains were prepared under septic conditions using Potato Dextrose Broth (PDB). These filtrates were prepared from 3 - 4 days sub-cultured *A. niger* and *Rhizopus sp*. on PDA. A piece of 9 mm in diameter of PDA for each fungal isolate was inoculated in a flask containing 25 mL PDB. Three sets of three flasks were assigned for *A. niger*, *Rhizopus sp.*, and control (Garuba et al., 2014; Hajieghrari, 2010) (Figure 1). The control flasks were inoculated with PDA only. After the inoculation, all flasks were incubated in the incubator at 25°C. Ten days later, the flasks were removed from the incubator, and the media was separated from the growth by filtration using sterile filter paper (Whatman glass microfiber filter paper Grade GF/F Retention 0.7 Micron).

In order to investigate the effect of fungal filtrates on the germination and growth of seedling of wheat grains, several clean and sterilized Petri dishes were prepared with sterilized filter paper. In each dish, five grains of wheat were sterilized with 0.1% hydrogen peroxide solution, and the grains were irrigation with 10 mL of the filtration. Three dishes containing the same number of wheat grains were used for each type of the filtrations. Two control plates were included in which one was irrigated with PDB, and another one was irrigated with sterile distilled water. All dishes were incubated in the incubator at 25°C. All plates were examined for the growth of grains on day three, five, seven, and ten. The percentage of germination was calculated according to the following equation:

\[
\text{Percentage of Germination} = \frac{\text{Number of Germinated Grains}}{\text{Total Number of Grains}} \times 100
\]
Effects of fungal filtrates on wheat grains germination
13

Statistical analysis
Statistical analysis was done using Graph Pad Prism version 5.01 (Graph Pad Software, USA). P values of <0.05 were considered significant. Error bars were expressed in the graphs as ± SD.

RESULTS AND DISCUSSION

Isolation and identification of fungi on wheat grains
It emerged from the current study that wheat grains obtained from the different production area were found to be contaminated with many types of fungi. A total of 319 colonies were isolated from wheat grains, of which 213 colonies were isolated from the Misurata Agricultural Research Center area, and 106 colonies were from the South Region of Libya. Table 1 shows that the number of colonies isolated from wheat grains from Misurata Agricultural Research Center area is more than the number of colonies isolated from the South Region of Libya. This variation in the number of colonies may be due to the Misurata Agricultural Research Center area has a moderate and humid climate during the sapling time, allowing the moisture-loving fungus and temperate fungi to have a more extended period of growing and reproduction for several generations, and thus producing a large number of germs. In contrary, the South Region of Libya is known for its relatively dry hot climate which could provide a short period of fungi on the cultivated grains to grow and reproduce during the time of the spikes production and consequently resulting in the decrease of production of germs. The results showed that Fusarium was predominant in wheat grains from southern Libya, which could be because the desert soils containing more Fusarium (Zeller et al., 2003). This fungus also can tolerate adverse conditions such as high temperatures and drought for long periods in the form of chlamedospores (Agrios, 2012; Cook, 1981). Furthermore, its dense presence in the desert soils, especially when cultivating sensitive species, contributes and increases the seriousness of the injury of plants at different stages which has negatively affects the quality and quantity of the crop (Zeller et al., 2003).

Table 1. The number of fungal colonies isolated from wheat grains obtained from Misurata Agricultural Research Center area and South Region of Libya.

<table>
<thead>
<tr>
<th>Isolated Fungi</th>
<th>Misurata Agricultural Research Center area</th>
<th>South Region of Libya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus sp.</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Aspergillus sp1</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Aspergillus sp2</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Aspergillus sp3</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Rhizopus sp.</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>Fusarium sp1</td>
<td>43</td>
<td>45</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td>Penicillium sp1</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Total number of colonies</td>
<td>213</td>
<td>106</td>
</tr>
<tr>
<td>Total number of species</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Total number of genera</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Morphological identification of fungal species was made using macroscopic and microscopic characterization. For fungi isolated from Misurata Agricultural Research Center area, ten species belonged to four genera, which are Aspergillus niger, Aspergillus sp., Aspergillus sp1, Aspergillus sp2, Aspergillus sp3, Rhizopus sp, Fusarium oxysporum, Fusarium sp1, Penicillium chrysogenum, and Penicillium sp1. Three fungal species belonged to two different genera which are Penicillium sp1, Fusarium sp1, and Fusarium sp were isolated from the South Region of Libya. Aspergillus niger and Rhizopus sp. (Figure 2) were the most common species isolated from wheat grains in both geographical areas. Thus, these genera were selected to investigate the effect of fungal filtrates on germination of wheat grains and seedling growth. These fungi were isolated from the grains and identified in Assiut University, Egypt.
**Effect of fungal filtrates on germination rate of wheat grains and seedling growth**

Wheat grains could be contaminated with fungi either in the field or during storage and exhibition in the markets. These fungi are likely to grow and produce toxins that may affect the vitality of the wheat grains. The role of fungal secretion on germination rates and development of seedlings of wheat grains has run out in this study. *A. niger* and *Rhizopus* sp. filtrates reduced the rate of germination after ten days to 20% and 80% respectively compared to germination rate of 100% for grains irrigated with distilled water as shown in Table 2 and Figure 3. This result was supported by a previous study, (Ibraheem et al., 1987) which found that *A. niger*, *A. flavus*, and *Alternaria alternata* had an inhibitory effect of reducing seed germination. These results are also consistent with many previous studies. Khokhar et al. reported that *P. chrysogenum* decreased the percentage of seed germination by 20.33%. This fungus had shown a poisoning effect on the seedling of cereal as evidenced in inhibition of the seed germination percentage of wheat at a higher percentage of 90% (Khokhar et al., 2013). The inhibition may indicate that the tested fungi produce toxic metabolites in the broth which they were cultured. *A. niger* and *P. chrysogenum* produced metabolites which are known to reduce germination and seedling development (Haikal, 2008). *A. niger* can produce mycotoxins such as oxalic acid crystals, kojic acid, and malformins depending on the growth condition and the strain of the organism (Garuba et al., 2014). This effect varies with different fungi.

Grains irrigated with *A. niger* filtrate showed a significant reduction of the radicle lengths (98.3% ± 0.6, 97.8% ± 0.5, 97.8% ± 0.2 and 97.8% ± 0.0) after three, five, seven and ten days respectively. This reduction rate is statistically significant when compared with irrigated grains with water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$), as shown in Figure 4. Again, grains irrigated with *Rhizopus* sp. filtrate showed a reduction of the radicle lengths (63.4% ± 3.7, 64.6% ± 4.6, 66.3% ± 4.2 and 67.4% ± 10.8) after three, five, seven and ten days respectively. This reduction rate is statistically significant when compared with irrigated grains with water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$) as shown in Figure 4. Similarly, the coleoptile lengths of grains irrigated with *A. niger* and *Rhizopus* sp. filtrates were also significantly shorter compared to grains irrigated with water (Two-Way ANOVA with Bonferroni post-test, $P < 0.001$) as shown in Figure 5. Grains irrigated with *A. niger* filtrate showed (93.9% ± 1.5, 95.5% ± 0.6, 95.3% ± 0.3 and 94.7% ± 1.9) reduction of the coleoptile lengths after three, five, seven and ten days respectively. Also, grains irrigated with *Rhizopus* sp. filtrate showed (43.5% ± 19.9, 43.2% ± 1.5, 36.0% ± 4.4 and 29.9% ± 4.7) reduction of the coleoptile lengths after three, five, seven and ten days respectively. These results are consistent with the finding of the previous study (Garuba et al., 2014). This study showed 65.33% and 79.67% germination ratio of corn seeds due to the effect of *A. niger* and *Penicillium chrysogenum* filtrates, respectively, when compared with control.

**Table 2. Effect of fungal on wheat grain germination.**

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<td><em>A. niger</em></td>
<td>20%</td>
</tr>
<tr>
<td><em>Rhizopus</em> sp.</td>
<td>80%</td>
</tr>
<tr>
<td>Control (PDA)</td>
<td>98%</td>
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<td>Control (Water)</td>
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**Figure 2.** Microphotographs of fungal species isolated from wheat grains. (A) *A. niger*, (B) *Rhizopus* sp.
Figure 3. Effect of fungi filtrates on the wheat grains germination and growth of seedlings after 10 days.

Figure 4. Effect of *A. niger* and *Rhizopus* sp. filtrates on the length of wheat radicles. A significant reduction of radicle lengths was observed when compared with grains irrigated with PDB and water (Two-Way ANOVA with Bonferroni post-test, *P* < 0.001). Results are expressed as mean ± SD from a representative experiment performed in triplicate.

Effects of fungal filtrates on wheat grains germination

Figure 5. Effect of *A. niger* and *Rhizopus* sp. filtrates on the length of wheat coleoptiles. A significant reduction of coleoptile lengths was observed when compared with grains irrigated with PDB and water (Two-Way ANOVA with Bonferroni post-test, \( P < 0.001 \)). Results are expressed as mean ± SD from a representative experiment performed in triplicate.

Results reported that *A. niger* filtrate has a more adverse effect on the germination rate of wheat grains and the development of their seedings. It could be due to the ability of the fungus to produce Aflatoxins. These findings are consistent with previous findings (Garuba et al., 2014; Jeswal, 1987; Tewfik et al., 1974) that concluded that Aflatoxins prevent or reduce the rate of germination of many crop seeds. It reduces the growth of the crop seeding as well as the development of the radicles and coleoptiles produced from irrigated grains.

Results also showed that *Rhizopus* sp. has a mild deleterious effect on wheat grain germination, seedling development, and evolution of the radicles and coleoptiles compared to *A. niger* which can be referred due to the differences in substrates and chemical quality from the toxins produced by *A. niger*. These toxins can also inhibit the naturally occurring Gibberellins produced by the embryo during germination leading to a shortening of the radicles and coleoptiles (Jones & Armstrong, 1971; Sinha, 1996).

Evaluation of the effect of *P. nigrum* (black pepper) extract on the growth of isolated fungi. The acetonic extract of *P. nigrum* showed significant inhibition effect on the growth of isolated fungi, *A. niger* and *Rhizopus* sp., from wheat grains, as shown in Figure 6 and Figure 7. The Inhibition rate of the treated fungal cultures with extract of *P. nigrum* were 85.7% ± 3.7 and 44.0% ± 3.1 for *A. niger*, and *Rhizopus* sp. respectively compared to untreated control (Paired t-tests with two-tailed, \( P < 0.001 \)). The inhibition effect of *P. nigrum* extract on *A. niger* and *Rhizopus* sp. may be due to it contains active compounds such as saponins, phenols, and alkaloids. It is known that these compounds have a powerful effect on many pathogens (Agrios, 2012; Gülçin, 2005; Pundir & Jain, 2010). The result of this study is consistent with previously published results (Abdel Mohsen, 2011). Many plant extracts, including black pepper, showed protection of sunflower plant from decay by *Macrophomina phaseolina*. These plants reduce the fungal infection rate by 53.1%. This inhibition by chili extract could be due to the presence of alkaloids capsorubin, dihydro, capsiain, and capsicin. This inhibition is also consistent with another study which showed the inhibition of the growth of pathogenic fungi, namely *Cochliobolus migabeanus*, *Pyricularia oryzae*, and *Rhizoctonia solani* in rice (Tewari & Nayak, 1991).

![Figure 6](image-url). Effect of *P. nigrum* extract on *A. niger* growth. (A) shows significant inhibition effect on the growth of isolated *A. niger* (Paired t-tests with two-tailed, \( P < 0.001 \)). Results are expressed as mean ± SD from a representative experiment performed in triplicate. The asterisk denotes the level of statistically significant differences from the untreated control. (B) shows colony morphology of *A. niger* treated with *P. nigrum*. (C) shows colony morphology of *A. niger* untreated with *P. nigrum*. 
project under University Seed Grant Number: SG-376-0216-IMS. The funders had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript. Thanks, are addressed to Misurata University, Misurata, Libya, and Misurata Agricultural Research Center for their cooperation in providing the essential information to complete this study.

REFERENCES

Abdel Mohsen, H. A. -J. 2011. Test the efficiency of some plant powders in the protection of the sun flower plant from the infection of the fungus Macrophomia phaseolina (tassi) Goid, which causes the disease. Faculty of Agriculture, University of Basra, 3(2).


Al Fadil, F. & Al Haidari, F. 2012. Effect of leachates isolated from rice residues on wheat grain germination and growth of Fusarium graminearum and Rhizoctonia solani nurses and Trichoderma harzianum. Department of Plant Protection - Faculty of Agriculture - University of Kufa Iraq.


CONCLUSION

The metabolic products of A. niger and Rhizopus sp impair the germination and growth rate of wheat and therefore diminishing its quality, especially the stored wheat grains. Hence necessity should be placed on these fungi and their metabolic processes due to their adverse effects on agriculture, health, and industry. Further studies on the biological control of fungi should be encouraged using other types of plants. The active compounds in these plants have the potential to be an effective antifungal agent.

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Ibrahim, M. A. O. 2015. Detection and identification of some seed borne fungi of groundnut from different locations with emphasis on *Ageriglal flavus* and its control in Sudan, Sudan University of Science and Technology.


Determination of potential solvents for novel N-substituted 5-(phenylamino)uracil derivatives and evaluation of their cytotoxic effects on Vero 76 Cells

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Abstract. N-substituted 5-(phenylamino)uracil derivatives have recently shown to possess potential antiviral properties. However, the high lipophilicity of these compounds has limited their ability to be dissolved in aqueous media for further in vitro and in vivo studies. This study aimed to determine the potential solvents for novel N-substituted 5-(phenylamino)uracil compounds and to evaluate the cytotoxic effects of these solvents on Vero 76 cells. Eight solvents, namely acetone, methanol, ethanol, dimethyl sulfoxide (DMSO), polyvinylpyrrolidone, nicotinamide, L-arginine, and sodium benzoate, were used to dissolve 1600 µM each of compound Z214 and compound Z276, which were chosen as the representatives of novel N-substituted 5-(phenylamino)uracil derivatives. Only L-arginine (700 mM), sodium benzoate (1500 mM), and DMSO (128 mM) were able to solubilise both compounds. Cytotoxicity assays on Vero 76 cells have shown that the maximum concentrations of L-arginine, sodium benzoate, and DMSO that demonstrated 100% cell viability were 108 mM, 10 mM, and 211 mM respectively. L-arginine at concentrations ranged from 215 mM to 860 mM have shown to significantly increased cell proliferation; while both sodium benzoate and DMSO have significantly reduced cell viability at concentrations ≥ 10 mM and ≥ 211 mM respectively. CC₅₀ values were 23.22 mM and 214.92 mM for sodium benzoate and DMSO respectively. The findings in this study revealed that DMSO at a concentration of 211 mM was found to be the most appropriate solvent to solubilise 1600 µM and below of novel N-Substituted 5-(phenylamino)uracil derivatives.

Keywords: N-substituted 5-(phenylamino)uracil derivatives, cytotoxicity, Vero cells, L-arginine, sodium benzoate, dimethyl sulfoxide

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INTRODUCTION

The solubility of the desired chemical compounds is considered as a fundamental process before the evaluation of their effectiveness as a potential drug takes place. The solubility of the compound is a state of the chemical substance called solute dissolved in solid, liquid or gaseous solvent to reach a homogeneous solution. This state is a crucial step as it plays an important role, particularly in testing the activity of the lipophilic compounds in vitro experiment due to the need to dissolve the compounds in hydrophilic media. There are several approaches available to improve the solubility of lipophilic agents. Common organic solvents such as methanol, ethanol, acetone, acetic acid, and dimethyl sulfoxide (DMSO) are widely used in the pharmaceutical industry. The addition of these solvents has shown to enhance the solubility of most of the lipophilic substances (Maes et al., 2012). Apart from that, more recent strategies in increasing the solubility of highly lipophilic substances include the use of mixed solvency concept, the addition of solubilizing enhancers or hydrotropic agents, combination with surfactants, development of solid dispersion, and nanosuspension (Maheshwari & Moondra, 2010; Savjani et al., 2012; Hamzeloo-Moghadam & Taiebi, 2014).

Hydrotropic solubilization technique is one of the potential tools to enhance the solubility of lipophilic substances. Hydrotropic agents are compounds that have amphiphilic molecular structures. The presence of amphiphilic molecular structures has shown to increase the solubility of poorly soluble solutes (Hodgdon & Kaler, 2007; Maheshwari & Moondra, 2010) through the interaction with water-soluble molecules via weak Van der Waals forces (Dhapte & Mehta, 2015). Hydrotropes which can be anionic, cationic or neutral, enhance the aqueous solubility of organic substances by forming aggregation, structure breaker and structure maker, as well as by developing micellar like structure (Dhapte & Mehta, 2015). Recent articles have shown that L-arginine, sodium benzoate, and nicotinamide have been used as hydrotropic agents to enhance drug solubility (Nidhi et al., 2011; Dhapte & Mehta, 2015). Arginine, which is an essential amino acid, serves as a precursor for many important molecules in cell biology. Apart from that, L-arginine has shown to have potential as a solubility enhancement agent (Shukla & Trout, 2010). The mechanism of arginine solubilizing properties is mostly associated with its ability to suppress the aggregation and adsorption of the proteins and other high molecular weight substances to the solid surface (Arakawa et al., 2007; Arakawa et al., 2008; Shukla & Trout, 2010; Shukla & Trout, 2011). Sodium benzoate, which is widely used as a preservative agent in food and pharmaceutical industries, also has shown the ability to solubilise hydrophobic agents (Maheshwari et al., 2009; Soni et al., 2014). It is also currently used as a cosolvent in mixed solvency approach to increase the solubility of some lipophilic drugs in aqueous solutions (Soni et al., 2014). Nicotinamide, which is a form of vitamin B3, is found in food and used as a dietary supplement and treatment for pellagra as well as a solubility enhancement agent (Rasool et al., 1991; Agrawal et al., 2004). It is used in the pharmaceutical industry to enhance the solubility of the drugs by forming a stacking complexation with the drug molecules (Kongmuang, 2002; Hussain et al., 1993). The drugs and the complex molecules interact with each other to reduce the exposure of the hydrophobic region to aqueous media (Sanghvi et al., 2007). Nicotinamide also has the ability to break the surface tension and water conductivity to form self-association (Sanghvi et al., 2007). Polyvinylpyrrolidone (PVP), a water-soluble polymer has been shown to serve as a solubilizing agent and function as a binder to the active ingredient to enhance the bioavailability of the drugs. In previous studies, PVP has shown to increase dissolution of the active ingredients and improve bioavailability of poorly water-soluble drugs such as indomethacin (Fini et al., 2008; Srikanth et al., 2010; Barmpalexis et al., 2013).

The non-nucleoside analogues are known to have broad-spectrum of antiviral activity. The chemical structure of most of the non-nucleoside analogues includes the presence of three aromatic rings connected by short linkers (Novikov et al., 2010). The newly synthesized N-substituted 5-(phenylamino)uracil derivatives are non-nucleoside analogues containing aromatic rings with the addition of uracil. The replacement of one aryl ring with uracil potentially offers several advantages such as a great potential of antiviral
activities against RNA viruses as well as advantages in pharmacokinetic properties (Novikov et al., 2010).

Novel uracil contained non-nucleoside analogues have demonstrated positive activity against HIV type 1, Hepatitis C virus (HCV), Epstein-Barr virus (EBV), and Human Cytomegalovirus (HCMV) (Maruyama et al., 2003; Novikov et al., 2010; Novikov et al., 2013). Novel 1-[ω-(phenoxy)alkyl]uracil derivatives (compounds 9–30) were shown to inhibit HCMV with EC₅₀ ranging from 8.9 µM to 100 µM (Novikov et al., 2013). The compound 13 from the same group of 1-[ω-(phenoxy)alkyl]uracil derivatives inhibited HIV-1(IIIB) and HIV-2(ROD) at 24 µM concentration with a 50% cytotoxic concentration of 154 µM (Novikov et al., 2013). In another study, 5-(arylamino)-1-benzyluracil derivatives have been shown to be effective against HCV (FH1 strain) with reducing of foci formation in Huh 7.5 cells at IC₅₀, approximately 20 µM (Awadh et al., 2013).

We intended to investigate the potential antiviral of novel N-substituted 5-(phenylamino)uracil derivatives against flavivirus. Considering the virus structure and protein functions similarities between HCV and dengue virus (DENV), we hypothesized that novel N-substituted 5-(phenylamino)uracil derivatives are also potentially active against DENV and other RNA viruses such as chikungunya virus (CHIKV) and zika virus (ZIKV) (Sun et al., 2005; Mosley et al., 2012; Sofia et al., 2012). However, the poor water-soluble properties of novel N-substituted 5-(phenylamino)uracil derivatives significantly limit their potential to be tested in vitro and in vivo experiments. This study aimed to determine the potential solvents for novel N-substituted 5-(phenylamino)uracil derivatives and to evaluate the cytotoxic effects of these solvents on Vero 76 cells.

**MATERIALS AND METHODS**

**Compounds and solvents**

Novel 1-[3-(phenoxy)benzyl]-5-(phenylamino)uracil and 1-[4-(phenoxy)benzyl]-5-(phenylamino)uracil derivatives were synthesized at the Department of Pharmaceutical Sciences, Medical University, Russia (Figure 1). Generally both groups of N-substituted 5-(phenylamino)uracil derivatives were prepared by adding a solution containing 6.16 mM of 3-(phenoxy)benzyl or 4-(phenoxy)benzyl bromide in 20 mL of 1,2-dichloroethane to a solution containing 5.98 mM of 2,4-bis(trimethylsilyloxy)-5-(arylamino) pyrimidine in 50 mL of 1,2-dichloroethane. The resulting mixture was refluxed for 14 h, cooled to room temperature, and then added with 10 mL ethanol. The precipitate was filtered and air-dried. The filtrate was evaporated by two thirds and cooled to 0°C. The resulting precipitate was filtered and combined with the previously obtained solid. This was followed by purification using column chromatography and eluting with ethyl acetate/CH2Cl2 (1:1), or by recrystallization from isopropanol/dimethylformamide (2:1). Only 2 compounds, compound Z214 (C25H23N3O3, MW 413.468) and compound Z276 (C27H27N3O3, MW 441.552), were used for solubility test as the representatives of the entire group. Acetone, methanol, ethanol, PVP, nicotinamide, L-arginine, sodium benzoate, and DMSO were chosen as the potential solvents for novel N-substituted 5-(phenylamino)uracil derivatives. All chemicals listed were obtained from Sigma-Aldrich, USA in analytical purity.

![Figure 1. General Structure Formula of (a): N-substituted 5-(phenylamino)uracil derivatives containing 4-(phenoxy)benzyl substituent at position 1 of uracil residue; (b): N-substituted 5-(phenylamino)uracil derivatives containing 3-(phenoxy)benzyl substituent at position 1 of uracil residue.](image-url)

**Dissolution of the tested compounds with the potential solvents**

Potential solvents were diluted in serial dilutions in order to find the minimal concentration capable to solubilise 1600 µM of the tested compounds. Dissolution of the tested compounds in different solvents was done under...
standard laboratory conditions and compounds were considered dissolved if the solution remained clear and did not reveal any signs of precipitations or sedimentation for at least 72 h.

**Cell cultures**

Vero (African green monkey kidney) 76 cells were obtained from the American Type Culture Collection (ATCC No: CRL1587). The cells were propagated in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 5% heat-inactivated Fetal Bovine Serum (FBS) (JRS, USA), 1% penicillin (100 units/mL) and 1% streptomycin (100 µg/mL) (HIMEDIA, India), and maintained at 37°C with 5% CO₂ (Rothan et al., 2014). Briefly, Vero 76 cells were cultured in a flask until 80% confluence. The cells were trypsinised, and the number of cells was counted. Vero cells 1×10⁴ in 100 μL DMEM supplemented with 1% of FBS were seeded into each well in 96 well plates and incubated for 24 h. Thereafter, the cells were divided into 4 groups. Group 1 was cultured in 1% DMEM (cell control), and groups 2 to 4 were cultured in 1% DMEM with a selected solvent at various concentration range. The concentrations for the solvents were ranged from 6.75 mM to 860 mM for L-arginine, 0.625 mM to 80 mM for sodium benzoate, and 35.5 mM to 1128 mM for DMSO. The cells were incubated for 72 h at 37°C with 5% CO₂. Morphological observation of treated and control cells was done daily using a fluorescent inverted microscope (IX81, Olympus, Japan). After 72 h incubation, MTS assay was performed to detect cell viability. Each concentration of the tested solvents was done in triplicates and the experiment was repeated 3 times.

**Cell viability assay**

Effects of L-arginine, sodium benzoate, and DMSO on Vero 76 cells viability were estimated using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS) assay (Promega, USA). MTS assay is a modification of tetrazolium reduction assay (MTT), which is a colorimetric assay for assessing cell viability. Tetrazolium assay determines the number of viable cells based on quantitation of metabolic activity in viable cells (Riss & Moravec, 2004). In the presence of NAD(P)H-dependent cellular oxidoreductases, yellow tetrazolium is reduced to purple formazan, the quantity of which is measured by recording the absorbance using a spectrophotometer. Briefly, after 72 h of incubation, 100 μL of media was removed from each well, and 20 μL of MTS was added to each well. The cells were further incubated for 1 h in the dark at 37°C with 5% CO₂. The absorbance was read at 490 nm with the reference wavelength of 630 nm using the plate reader (Tecan Saphire) (Lani et al., 2015). The percentage of cell viability (CV) was calculated using the formula as below:

\[
CV = \frac{\text{Average absorbance of triplicate of drug wells}}{\text{average absorbance of control wells}} \times 100\%
\]

Percentages of cell viability above 80% were considered as non-cytotoxicity; within 80% - 60% weak; 60% - 40% moderate, and below 40% as a strong cytotoxicity (International Organization for Standardization, 2009). Percentages of cell viability above 110% were considered as cell proliferative effect.

**CC₅₀ determination**

The value of 50% cytotoxic concentration (CC₅₀) on Vero 76 cells for each of the tested solvent was calculated based on cell viability data using dose-response curves plotted in Sigma Plot Software (version 12.0). Firstly, the data were expressed as normalized values of three values of the percentage of cell viability from three independent experiments. After all the values (experimental points) were placed on the graph, the approximation curve was selected. The choice of a particular curve was done based on two criteria: (i) the appearance of the graph (the location of the approximation curve relative to the experimental points); and (ii) the value of the correlation coefficient R that has to be closer to 1. When the approximation curve was selected, the coefficients b and c were determined, and CC₅₀ was calculated based on the equation below:

\[
CC_{50} = -\frac{1}{d} \ln \frac{0.5}{c}
\]

**Statistical analysis**

The experiment was performed in triplicates and repeated at least three times. The results were expressed as the mean ± standard deviation. One-way ANOVA test was performed to compare the
cell viability of each tested concentration against the control. P-value of less than 0.05 was considered as statistically significant.

RESULTS

Solubility of N-substituted 5-(phenylamino)uracil derivatives in different solvents

Several potential solvents were tested to solubilize novel N-substituted 5-(phenylamino)uracil derivatives. Acetone, methanol, ethanol, PVP, and nicotinamide failed to solubilize tested compounds at the concentration of 1600 µM. They did not solubilize 5-(phenylamino)uracil derivatives completely, and the addition of culture medium further resulted in a turbid solution. Whereas, L-arginine, sodium benzoate and DMSO have successfully dissolved 1600 µM of both tested compounds at a minimum concentration of 700 mM, 1500 mM, and 211 mM respectively.

Effect of solvents on Vero cells morphology

Observation of Vero 76 cell morphology in the presence of solvent at 72 h showed that cells exposed to the highest tested concentrations of sodium benzoate and DMSO displayed shrinkage and rounding shape. The cellular detachment was also observed in cells treated with the highest tested concentrations of sodium benzoate and DMSO (Figure 2). Cells treated for 72 h with 860 mM of L-arginine revealed signs of cell proliferation, such as increasing of cell number, cell stacking, and multilayer formation. There were no morphological changes observed on non-treated (cell control) cells or Vero cells exposed to low concentrations of the tested solvents after 72 h of incubation (Figure 2).

Figure 2. Effects of solvents on Vero 76 cell morphology at 72 h post exposure observed by light microscopy (Magnification 10x10). (a), (e), (i): Cell control; (b): L-arginine 6.75 mM; (c): L-arginine 108 mM; (d): L-arginine 860 mM; (f): Sodium benzoate 1.25 mM; (g): Sodium benzoate 10 mM; (h): Sodium benzoate 80 mM; (j): DMSO 35.5 mM; (k): DMSO 211 mM; (l): DMSO 564 mM. The sites of cell shrinkage, rounding shape, and detachment are marked by arrows.
Effects of solvents on cells viability
The cytotoxic effects of L-arginine, sodium benzoate, and DMSO on Vero 76 cells after 72 h of exposure were evaluated using MTS assay. L-arginine did not affect cell viability at concentrations ranged from 6.7 mM to 108 mM, revealing 107.5 ± 2.87% of cell viability (Figure 3). However, at concentrations ranged from 215 mM to 860 mM, a dramatically increased cell proliferation up to 180% was observed (Figure 3). Sodium benzoate at concentrations from 0.625 mM to 10 mM was able to maintain cell viability close to 100%. On the contrary, the exposure of the concentration above 20 mM resulted in a significant dose-dependent reduction in cell viability (Figure 4). DMSO showed no cytotoxic effect at concentrations ranged from 35.5 mM to 211 mM, maintaining the cell viability from 98.11 ± 3.66% till 106.40 ± 2.87% (Figure 5). Nonetheless, an increasing concentration of DMSO more than 211 mM was associated with a significant reduction of cell viability (Figure 5).

![Figure 3. Effect of L-arginine on Vero 76 cell viability after 72 h incubation. Each bar represents the mean of percentage of viable cells ± SE. *p-value < 0.05 vs cell control.](image)

![Figure 4. Effect of sodium benzoate on Vero 76 cell viability after 72 h incubation. Each bar represents the mean of percentage of viable cells ± SE. *p-value < 0.05 vs control, ***p-value < 0.001 vs cell control.](image)
Figure 5. Effect of DMSO on Vero 76 cell viability after 72 h incubation. Each bar represents the mean of percentage of viable cells ± SE. ***p-value < 0.001 vs cell control.

**CC_{50} determination**

CC_{50} for sodium benzoate and DMSO were calculated by nonlinear regression (Figure 6, Figure 7). CC_{50} values for sodium benzoate and DMSO were determined as 23.22 mM and 214.92 mM respectively, indicating sodium benzoate is more cytotoxic to Vero 76 cells compared to DMSO (Table 1).

**Table 1.** CC_{50} of L-arginine, Sodium benzoate and DMSO on Vero 76 Cells after 72 h of exposure.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>R</th>
<th>CC_{50} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine</td>
<td>-</td>
<td>&gt; 860</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>0.97</td>
<td>23.2233</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.95</td>
<td>214.9229</td>
</tr>
</tbody>
</table>

R - correlation coefficient

Figure 6. Determination of CC_{50} of sodium benzoate on Vero 76 cells using SigmaPlot Software. The data are expressed as normalized values of three independent experiments. CC_{50} of sodium benzoate is 23.22 mM
DISCUSSION

The newly synthesized N-substituted 5-(phenylamino)uracil derivatives are non-nucleoside uracil analogues with high potential to be developed as antiviral drugs against RNA viruses (Novikov et al., 2010). Being highly lipophilic substances, these compounds have some limitations for further in vitro studies as the cells are maintained in aqueous media. Vero 76 cells are a continuous cell lineage, and it is one of the most common mammalian cell lines used for virology research. Vero cells do not produce interferon and, therefore, this cell line is one of the best in vitro models for testing of new antiviral drugs (Emeny & Morgan, 1979; Matskevich et al., 2009). Vero 76 cells are also used to produce both live and inactivated poliovirus, rotavirus, smallpox, etc. vaccines, as well as for propagation of different viruses including Rabies virus, Reovirus, and Japanese encephalitis virus (Barrett et al., 2009; Murray et al., 2017). Apart from virology studies, Vero 76 cells are widely used for propagation of intracellular bacteria and parasites (Singh et al., 2013). Thus, testing the cytotoxic effect of different solvents on Vero cells provides useful data not only in virology but also benefits bacteriology and parasitology.

In this study, three solvents namely L-arginine, sodium benzoate and DMSO, were able to dissolve 1600 µM of Z214 and Z276 compounds to produce a stock concentration for further in vitro studies on Vero 76 cells. Based on the cytotoxic activity of 5-(arylamino)-1-benzyluracil derivatives on CEM SS cells, which included compound 11 with CC$_{50}$ of 168 µM, compounds 2, 3, 8, 12, 13 (CC$_{50}$ > 100 µM) and compounds 4, 5 (CC$_{50}$ > 200 µM) (Novikov et al., 2010), the stock concentration of 1600 µM for both Z214 and Z276 were chosen as it allowed further testing of cytotoxicity and antiviral effects at concentration range up to 800 µM. Since these solvents were able to solubilize the tested compounds, the determination of their toxicity on Vero 76 cells is considered important as ideally, a solvent should not cause any undesired effect to the tested cells. The determination of the cytotoxicity of these solvents on Vero 76 cells was performed at 72 h of exposure. Considering novel N-substituted 5-(phenylamino)uracil derivatives to be screened against RNA viruses and primarily against DENV, the choice of 72 h was based on the peak post-infection titers of DENV in Vero cells as described in Lang et al., 2016. The

Figure 7. Determination of Cytotoxicity of DMSO on Vero 76 cells using SigmaPlot Software. The data are expressed as normalized values of three independent experiments. CC$_{50}$ of DMSO is 214.92 mM.
cytopathic effect of ZIKV in C6/36 and Vero cell cultures was also found to be more evident at 72 h post-infection (Barreto-Vieira et al., 2017).

L-arginine is known to have low toxicity for cell culture. In our current study, L-arginine did not reveal any cytotoxic effects at the tested concentrations. However, it appeared to enhance the growth of Vero 76 cells at concentrations from 215 to 860 mM that correlates with the previously reported study, which showed that human endometrial RL-95-2 cells (Greene et al., 2013). L-arginine also stimulated proliferation and prevented the lipopolysaccharide-induced death of intestinal cells (Tan et al., 2010). The presence of L-arginine-induced proliferative effect can complicate the assessment of antiviral activity of potential antiviral compounds and makes L-arginine an unsuitable solvent for future testing of in vitro antiviral activity on Vero 76 cells. On the other hand, the ability of L-arginine to stimulate cell proliferation could suggest the potential role of L-arginine as a wound-healing agent as discussed in a review by Alexander and Supp, 2014 (Alexander & Supp, 2014). In other studies, L-arginine also has shown to have anti-aging effect associated with some advance in the immune response and reduced risk of cardiovascular diseases (Gokce, 2004; Gad, 2010).

Sodium benzoate at the concentration of 20 mM was shown to produce a weak cytotoxic effect (73 ± 7.49% of cell viability), that was corresponding to the concentration of 2 mg/ml that had been found to increase chromosomal break and mutagenic effect in lymphocytes (Pongsavee, 2015). However, the cells demonstrated a dramatic decline in viability at doses of more than 20 mM. Due to the relatively high cytotoxicity on Vero 76 cells, particularly at higher concentrations, sodium benzoate was also excluded as an appropriate solvent for the novel N-substituted 5-(phenylamino)uracil derivatives.

DMSO is one of the common solvents used in the pharmaceutical industry. DMSO is characterised by low viscosity and has a high solubilizing capacity for polar and non-polar compounds (Da Violante et al., 2002; Galvao et al., 2014). It is commonly used to solubilize hydrophobic drugs in cell culture studies. However, the data regarding DMSO cell culture toxicity is quite controversial and dependent on the type of cells. Galvao et al. (2014) reported DMSO did not produce a toxic effect on retinal ganglion cells at the concentrations of less than 10% (Galvao et al., 2014). The study on Caco2/TC7 cells also has shown that DMSO was non-toxic at the concentrations up to 10% (Da Violante et al., 2002). While other studies have shown that only the concentrations of 1% or lower are non-toxic (Sharma et al., 2014). In our current study, we also have found that DMSO was able to maintain cell viability on Vero 76 cells at the concentration of 211 mM which corresponds to 1.5% solution.

We accept that these results are limited for Vero 76 cells only and for completeness of the study, it is necessary to test the cytotoxic effect of these solvents on other cell lines commonly used in virology, such as Huh-7, BHK cells, and HEK-293 cells.

CONCLUSION

Based on the results of this study, we conclude that DMSO at the concentration of 211 mM (1.5%) is the most suitable agent to solubilize N-substituted 5-(phenylamino)uracil derivatives for further screening of potential antiviral activity against RNA viruses on Vero cells in vitro.

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REFERENCES


Analysis of normalization method for DNA microarray data

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Abstract. Normalization is a process of removing systematic variation that affects measured gene expression levels in the microarray experiment. The purpose is to get more accurate DNA microarray result by deleting the systematic errors that may have occurred during the making of DNA microarray Image. In this paper, five normalization methods of Global, Lowess, House-keeping, Quantile and Print-tip are discussed. The Print Tip normalization was chosen for its high accuracy (32.89 dB and its final MA graph shape was well normalized. Print tip normalization with PSNR value of 33.15dB has been chosen as a new normalization method. The results were validated using four images from the formal database for DNA microarray data. The new proposed method showed more accurate results than the existing methods in term of four parameters: MSE, PSNR, RMSE and MAE.

Keywords: background, DNA, Global, house-keeping, Lowess, microarray, normalization, Print-tip, Quantile

INTRODUCTION

Gene expression measurements provide clues about the regulatory mechanism, biochemical pathways and broader cellular function. By gene expression, it can be understood as the transformation process of gene's information into proteins. The formal transformational pathway of protein begins from DNA (deoxyribonucleic acid) which is copied to the mRNA (messenger ribonucleic acid) and, finally, this molecule passes from nucleus to cytoplasm carrying the information to build up proteins (Schena, 1999).

There are many microarray analysis software packages available on the market whether commercial or freeware. Basically, each software program can be separated into three main tasks: (1) gridding or addressing, which is the process of specifying coordinate to every spot on the slide, (2) the segmentation which decides the classification of each pixel either as foreground which corresponds to be an interesting spot or as background which acts as an error or noise, (3) the Intensity Extraction which is the step to calculate green and red for foreground fluorescence intensity for each spot on the array (Monica et al., 2011; Youlan et al., 2008; Hovatta et al., 2005). Subsequently, there are many processes to inspect the results and also to correct the errors that have occurred. The background correction method which ignores the effect of intensity of the background. This can be achieved by subtracting the value of the background intensity from the value of foreground intensity or any other suitable method to neglect the effect of background intensity. Another process to increase the accuracy is the normalization method which we are going to discuss in this paper (Yang et al.,

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Normalization is a process of removing systematic variations that affect measured gene expression levels in microarray experiments. The purpose of normalization is to adjust for effects which arise from variations in the microarray technology rather than from biological differences between the RNA samples or between the printed probes. Imbalances between the red and green dyes may arise from differences between the labeling efficiencies or scanning properties of the two flours complications perhaps by the use of different scanner settings (Geeleher et al.; Karakach et al., 2010). The aim of the paper is to review and make some comparison between various methods in microarray data normalization.

In Literature Review, Section A discusses the purpose of normalization. In addition, several normalization algorithms are discussed in Section B, while Section C discusses the comparison of the different methods. Then, this article represents methodology and results of each method and finally summarizes the article in the conclusion.

**Literature Review**

**A. Purpose of normalization and normalization expression graphs**

The purpose of normalization is to adjust for effects which occur from variations in the microarray technology rather than from the biological differences between the RNA samples or between the printed probes. It can be regarded as a sort of calibration process that improves the comparability among microarrays treated alike. Imbalances between the red and green intensities may arise from differences between the labeling efficiencies or scanning properties of the two slides which may due to the use of different scanner settings. If the imbalance is more complicated than a simple scaling of one channel relative to the other, then a function of normalization will need to be performed. As an example of the importance of the normalization process, by comparing Figure 1 with Figure 2, a different was observed once the background correction is ignored. Figure 1 represents M-A plot for a red and green intensity before correcting the background values, thus, it shows irregular distributions of the spot around the plot. However, there is a spot regulation in Figure 2 for normalized intensities. For more details refer to (Adriaens et al., 2012; Babu Madan, 2004).

![Figure 1. M-A Plot for No-Background Corrected Slide (Adriaens et al., 2012).](image1)

![Figure 2. M-A Plot for Background Corrected Slide (Adriaens et al., 2012).](image2)

**B. Normalization graph expression**

Normalization can be expressed in two types of graphs. First one is the logarithm of the red intensity versus the logarithm of the green intensity (log R vs. log G) as shown in Figure 3. The second one is M-A plot, it is 45° rotation of standard scatter plot as shown in Figure 4. Write R (Red intensity) and G (Green intensity) for the
background-corrected red and green intensities for each spot, normalization is usually applied to the log-ratios of expression, which will be written as in Equation (1). The mean of log-intensity of each spot will be written as in Equation (2), a measure of the overall brightness of the spot. (The letter M is a mnemonic for minus while A is a mnemonic for addition) (Dudoit et al., 2002).

\[ M = \log R - \log G \]  
\[ A = (\log R + \log G)/2 \]

Figure 3. Log R vs. Log G

Figure 4. M-A Plot.

C. The latest trend in microarray normalization

There are many studies on the DNA microarray normalization. As a result, many methods were created and their results were drawn as M-A plot or any other type of plots representation. This section will discuss and elaborate these methods in order to choose the most suitable one and develop it for further microarray analysis.

The first method is Global normalization, the underlying assumption of this approach is that the total of mRNA labeled with either R-value (sum of red intensities) or G value (sum of green intensities) is equal. While the intensity for any spot may be higher in one channel than the other, when averaged over thousands of spots in the array, these fluctuations should average out. Consequently, in this method, it takes the value of c out of a log (R/G). The c value is equal to the main assumption that equal to the log of the total R (Red intensity) over total G (Green intensity) which can be expressed by the variable K, Equation (3) and (4) explain this method (Yang et al., 2002).

\[ \log_2 (R/G) \rightarrow \log_2 (R/G) - c = \log_2 (R/(kg)) \]  
\[ K = \sum (R/G) \]  

House-keeping method is a similar method that uses a fixed value to subtract or add to the (M) value. However, this method requires a specific gene call house-keeping gene. The expression of the house-keeping gene is assumed to be constant. Therefore, after hybridization, the intensity of these genes is identified and the difference should be calculated which would be used later for normalizing the other genes (Bilban et al., 2002).

The intensity-dependent normalization (Lowess) runs a line through the middle of the MA plot, shifting the M value of the pair (A, M) by \( m = \text{mean} (M) \), as shown in Equation (5). One estimate of \( m \) is made using the Lowess function (Locally Weighted Scatterplot Smoothing). As in Figure 5 and Figure 6, the difference between Global and Lowess normalization can be noticed in M-A plot form (Berger et al., 2004).

\[ \log_2 (R/G) \rightarrow \log_2 (R/G) - m = \log_2 \left( \frac{R}{(M \text{Number of Spots/G})} \right) \]  

Figure 5. Global normalization (Berger et al., 2004).
The Print-tip normalization is similar to Lowess normalization that repeating itself in groups, where each group is separated than the others. Thus, Print Tip normalization starts by dividing a value \((\log R + \log G)/2\) into tip groups. Then, each group is normalized by subtracting its M-value \((\log R - \log G)\) from its corresponding value \((\text{Lowess}(A))\) of the tip group as in Equation (2). This value \((\text{Lowess}(A))\) is equal to the mean of M value inside each tip group. The normalized log-ratios \((N)\) will replace the M values to restore back the red and green intensities. A simpler form of Print-tip is shown in Equation (6) where \((\text{Lowess}(A))\) is the global Lowess curve plotted in Figure 7. Refer to Figure 8 for the final figure of the Print-tip normalization (Smyth et al., 2003).

\[
N = M - \text{Lowess}(A)
\]  

Lastly is the Quantile normalization method which is also one of the most favorable approaches used especially in normalization between arrays. First, rearrange the genes in each column as in the second table in Figure 8. Then, take the mean in each row and replace the whole row by the mean value as in the third table in Figure 8. Finally, reorder each gene in its original place with its new value (Yang et al., 2001).

D. Comparison of different Normalization approaches

In this section, the existing system algorithm as discussed in section will be analyzed and discussed to find out the similarities and variations among the different normalization methods. Table 1 summarized the comparison of these algorithms.

From Table 1, it can be seen that all methods used are mainly the value of M which equal to log of red intensity minus log of green intensity. However, three methods have different value to subtract from M. To illustrate, Global normalization use the log of the addition of each of red and green intensity while the other two methods are using median and global median.

In term of the final shape of the normalization on M-A graph, there are similarities between Lowess and Print-tip methods because both have a straight median line in the value of \((M = 0)\) due to their similarities on subtracting the mean or median from M. However, in Global normalization, there is a curve around the value of \((M= 0)\) due to the subtraction of the total R and G.
Table 1. Comparison between different system algorithms.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Method</th>
<th>Function</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Yang et al., 2012)</td>
<td>2012</td>
<td>Global</td>
<td>Log (R/KG)</td>
<td>K = sum(R) / sum(G)</td>
</tr>
<tr>
<td>(Berger et al., 2004)</td>
<td>2004</td>
<td>Lowess</td>
<td>Log (R/G) – C(A)</td>
<td>LOWESS Function</td>
</tr>
<tr>
<td>(Martin et al., 2002)</td>
<td>2002</td>
<td>House Keeping</td>
<td>N = M – Housekeeping value</td>
<td>House Keeping</td>
</tr>
<tr>
<td>(Smyth &amp; Speed, 2013)</td>
<td>2013</td>
<td>Print Tip</td>
<td>N = M – Lowess(A)</td>
<td>Global Lowess</td>
</tr>
<tr>
<td>(Adriaens et al., 2012)</td>
<td>2012</td>
<td>Quantile</td>
<td>Means of rows after reorder</td>
<td>NA</td>
</tr>
</tbody>
</table>

House-keeping and Quantile normalization methods do not use M-A plot, consequently, their final graphs do not always take a straight line of the mean on the (M=0). In addition, house-keeping requires knowing the expected intensity value of its genes to compare it with the final intensity value; therefore, it is difficult to examine it in this project. The main reason for testing the house-keeping method because it is one of the main and most commonly use fixed normalization type methods.

According to this review, we suggest Print-tip normalization method to be used because when comparing to the global normalization, its final figure is simpler and easier to read, and can also be compared easily to various plots. A straight line (M=0) is easier to read than the Global normalization curve. However, when it is compared to loess normalization’s final figure, there was not much different in the value of M after the normalization and thus, in the end, the M value is noticeable (Smyth et al., 2003).

E. Results validation parameters

In order to choose the most accurate method, as discussed by (Chaurasia et al., 2015), four parameters were used to compare each method results with the Princeton results. These parameters are:

1. **MSE (Mean square Error):** is defined as some sort of average or sum (or integral) of the square of the error between two intensities as in Equation 3.

   \[
   \text{MSE} = \frac{1}{(N^*M)} \times \sum (x(i,j) - y(i,j))^2
   \]  
   
   (7)

   Where: \(x(i, j)\) is the original intensity from Princeton, \(Y(i, j)\) is the intensity for a specific method, \(M\) and \(N\) are the dimensions of the image.

2. **Peak Signal to Noise Ratio (PSNR):** defined as the ratio between signal variance and reconstruction error variance as in Equation 4.

   \[
   \text{PSNR} = 20 \times \log (\text{max}) - 10 \times \log (\text{MSE})
   \]  
   
   (8)

   Where mean squared error (MSE) and max is the maximum possible pixel value of the intensity.

3. **RMSE (root mean square error):** is defined as the square root of mean square error as in Equation 5.

   \[
   \text{RMSE} = \sqrt{\text{MSE}}
   \]  
   
   (9)

4. **MAE (maximum absolute error):** is defined as the maximum absolute value, the difference between Princeton intensity and one of the reviewed methods as in Equation 6.

   \[
   \text{MAE} = \text{Max} (|x(i,j) - y(i,j)|)
   \]  
   
   (9)

   In this work, Matlab version R2013a 9.0 and its Image Processing Toolbox which supports an extensive range of image processing operations are used for data analysis and technical computing due to its high performance and powerful language. This work is implemented using a personal computer with a processor: Intel (R) Core (TM) i3 -1.80 GHz.

**METHODOLOGY**

Following the previous steps and in order to examine the suitable method which would provide more accurate algorithm among normalization algorithms that was reviewed in Literature Review section, four DNA microarray images were used as shown in Figure 9, these images are from Princeton University microarray...
database. The formula codes were applied according to normalization methods that have been discussed in Section two. These normalization methods are Global normalization, Lowess normalization, Print Tip normalization and Quantile normalization. However, housekeeping normalization will not be examined because it requires house-keeping gene from the manufacturer. Princeton University microarray database provide the measured intensity information for each image. Thus, this information was used as a reference to compare and validate this research results according to four parameters, these parameters are MSE, PSNR, RMSE and MAE. For an overview of the parameters, the reader should refer to Literature Review section.

\[ PT_{\text{new}} = \frac{(m+PT(i))}{2} \]  
\[ Rn = \sqrt{2^{(2a+m)}} \]  
\[ Gn = \sqrt{2^{(2a-m)}} \]

\( \text{(11)} \)  
\( \text{(12)} \)  
\( \text{(13)} \)

B. Result's validation

In terms of four parameters, MSE, PSNR, RMSE and MAE, the new normalization algorithm validated by comparing its results with the existing algorithm using Princeton results as a reference for the four images in Figure 9, these images are real pictures obtained from a public database of the Princeton University microarray database. It is important to notice that the databases calculations are in a form of 16 bits while the algorithm that used in this work is in 8 bits. Therefore, it is compulsory to change the databases to 8 bits forms before comparing it with this project’s result. In this section, it is also important to notice that the red and green intensity will not be considered. Therefore, the error will be calculated for 200 spots regardless of the colors whether it is red or green.

RESULTS AND DISCUSSION

This work discusses five methods for normalization applied on four DNA microarray images in Figure 9. The spots intensity results for these methods were compared with the Princeton databases. The comparison was done depending on four basic parameters. These parameters are PSNR (Peak Signal to Noise Ratio), MSE (Mean Square Error), RMSE (Root Mean Square Error) and MAE (Maximum Absolute Error) as they were discussed in Literature Review section.

From these tables, Table 2, Table 3, Table 4 and Table 5, it can be seen that the new proposed algorithm gave more accurate results especially in term of MAE in all the tables. However, in terms of MSE, PSNR and RMSE, the new algorithm was
the best algorithm in Table 2 and Table 3 only. To illustrate, Print Tip showed the best results regarding these three parameters in Table 3 while Lowess normalization was the best in Table 1. However, the Quantile normalization gave huge amount of error.

PSNR (Peak Signal to Noise Ratio) for normalization presents much lower results than the PSNR results for intensity extraction as in Literature Review section by around 10 dB units for each image. This is because the normalization comes after intensity extraction, so the noises that were appeared during the intensity extraction, it also would be included for normalization.

### Table 2. Accuracy of normalization methods applies on Princeton image (a) in Figure 9.

<table>
<thead>
<tr>
<th>Method</th>
<th>MSE</th>
<th>PSNR</th>
<th>RMSE</th>
<th>MAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>39.24</td>
<td>32.23</td>
<td>6.26</td>
<td>13.23</td>
</tr>
<tr>
<td>Lowess</td>
<td>43.05</td>
<td>31.83</td>
<td>6.56</td>
<td>14.00</td>
</tr>
<tr>
<td>Quantile</td>
<td>1444.03</td>
<td>16.57</td>
<td>38.00</td>
<td>168.37</td>
</tr>
<tr>
<td>Print Tip</td>
<td>38.36</td>
<td>32.33</td>
<td>6.19</td>
<td>15.84</td>
</tr>
<tr>
<td>New</td>
<td>36.40</td>
<td>32.55</td>
<td>6.03</td>
<td>12.21</td>
</tr>
</tbody>
</table>

### Table 3. Accuracy of normalization methods applies on Princeton image (b) in Figure 9.

<table>
<thead>
<tr>
<th>Method</th>
<th>MSE</th>
<th>PSNR</th>
<th>RMSE</th>
<th>MAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>37.82</td>
<td>32.39</td>
<td>6.15</td>
<td>17.02</td>
</tr>
<tr>
<td>Lowess</td>
<td>60.28</td>
<td>30.36</td>
<td>7.76</td>
<td>24.74</td>
</tr>
<tr>
<td>Quantile</td>
<td>1670.65</td>
<td>15.94</td>
<td>40.87</td>
<td>171.00</td>
</tr>
<tr>
<td>Print Tip</td>
<td>36.61</td>
<td>32.53</td>
<td>6.05</td>
<td>16.44</td>
</tr>
<tr>
<td>New</td>
<td>41.61</td>
<td>31.97</td>
<td>6.45</td>
<td>13.57</td>
</tr>
</tbody>
</table>

### Table 4. Accuracy of normalization methods applies on Princeton image (c) in Figure 9.

<table>
<thead>
<tr>
<th>Method</th>
<th>MSE</th>
<th>PSNR</th>
<th>RMSE</th>
<th>MAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>31.15</td>
<td>33.23</td>
<td>5.58</td>
<td>14.67</td>
</tr>
<tr>
<td>Lowess</td>
<td>33.41</td>
<td>32.93</td>
<td>5.78</td>
<td>13.67</td>
</tr>
<tr>
<td>Quantile</td>
<td>1219.27</td>
<td>17.30</td>
<td>34.92</td>
<td>135.21</td>
</tr>
<tr>
<td>Print Tip</td>
<td>32.86</td>
<td>33.00</td>
<td>5.73</td>
<td>15.55</td>
</tr>
<tr>
<td>New</td>
<td>30.75</td>
<td>33.29</td>
<td>5.55</td>
<td>10.80</td>
</tr>
</tbody>
</table>

### Table 5. Accuracy of normalization methods applies on Princeton image (d) in Figure 9.

<table>
<thead>
<tr>
<th>Method</th>
<th>MSE</th>
<th>PSNR</th>
<th>RMSE</th>
<th>MAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>22.01</td>
<td>34.74</td>
<td>4.69</td>
<td>14.25</td>
</tr>
<tr>
<td>Lowess</td>
<td>21.35</td>
<td>34.87</td>
<td>4.62</td>
<td>15.25</td>
</tr>
<tr>
<td>Quantile</td>
<td>2527.36</td>
<td>14.14</td>
<td>50.27</td>
<td>254.97</td>
</tr>
<tr>
<td>Print Tip</td>
<td>27.85</td>
<td>33.72</td>
<td>5.28</td>
<td>16.18</td>
</tr>
<tr>
<td>New</td>
<td>21.76</td>
<td>34.79</td>
<td>4.67</td>
<td>11.18</td>
</tr>
</tbody>
</table>

These findings support the finding of (Smyth et al., 2003) as he mentioned that the “Print-Tip Lowess normalization provides a well-tested general purpose normalization method which gives good results on a wide variety of arrays”. It is best combined with diagnostic plots of the data. When the diagnostic plots show that biases still remain in the data after normalization, further normalization steps such as house-keeping or quantile normalization between the arrays may be undertaken. Besides that, the new algorithm represented the most accurate results than all other existing methods.

### CONCLUSION

In this paper, Normalization is defined as a process to delete systematic error which is why it is important and necessary. Since there are many normalization methods that exist, five most commonly used normalization algorithms such as Global, Lowess, House-keeping, Quantile, and Print-tip have been tested and compared to find the most suitable approach in a general normalization process. For that purpose, a Matlab code was built for each method for two slides; the ideal and real microarray slides. The results were shown in two forms, Table of red and green intensities and M-A graph. The results show that Global, Lowess, and Print-tip have more accurate result once compared with an ideal image result while Print-tip has the advantages than the other two especially in term of final graph shape. By combining Lowess and Print Tip normalization, a new algorithm for normalization was proposed and applied on four DNA microarray image from Princeton website. Using Princeton results, this new algorithm was compared with the existing algorithms; the results validate this algorithm as one of the best algorithms for DNA microarray normalizations.

### ACKNOWLEDGEMENT

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REFERENCES


Plasmodial enzymes in metabolic pathways as therapeutic targets and contemporary strategies to discover new antimalarial drugs: a review

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Abstract. Malaria continues to pose imminent threat to the world population, as the mortality rate associated with this disease remains high. Current treatment relies on antimalarial drugs such as Artemisinin Combination Therapy (ACT) are still effective throughout the world except in some places, where ACT-resistance has been reported, thus necessitating novel approaches to develop new anti-malarial therapy. In the light of emerging translational research, several plasmodial targets, mostly proteins or enzymes located in the parasite’s unique organelles, have been extensively explored as potential candidates for the development of novel antimalarial drugs. By targeting the metabolic pathways in mitochondrion, apicoplast or cytoplasm of \textit{Plasmodium}, the possibility to discover new drugs is tremendous, as they have potentials as antimalarial therapeutic targets. This literature review summarizes pertinent information on plasmodial targets, especially enzymes involved in specific metabolic pathways, and the strategies used to discover new antimalarial drugs.

Keywords: \textit{Plasmodium} enzymes, metabolic pathways, antimalarial target, drug discovery

INTRODUCTION

Despite the reduced numbers of malaria cases in the world, the morbidity and mortality rates remain disturbing. The World Health Organization (WHO) in 2017, reported that 219 million malaria cases have been registered worldwide. From this number, almost 92\% of the cases occurred in WHO African Region, WHO South-East Asia Region (5\%) and the Eastern Mediterranean (2\%) regions (WHO, 2018). Malaria is caused by the parasites from genus \textit{Plasmodium}, where infected \textit{Anopheles} mosquitoes play a role as the transmission vector. Human infection with \textit{Plasmodium} is normally associated with four species; specifically, \textit{Plasmodium falciparum}, \textit{P. vivax}, \textit{P. malariae} and \textit{P. ovale}. \textit{P. ovale} that caused malaria in human can be divided into two distinct \textit{ovale} malaria species, which are \textit{P. ovale curtisi} and \textit{P. ovale walkeri}, after a study conducted by researchers from Mahidol University, Bangkok and UK Malaria Reference Laboratory, where polymorphisms in six loci were observed in 55 isolates. It was found that two diverse major haplotypes of each locus were identified and these did not recombine in any of the parasites examined (Alemu et al., 2013; Rowe et al., 2006; Sutherland et al., 2010).

The recent distribution of \textit{Plasmodium} species shows that majority of \textit{P. falciparum} infection...
occurred in tropical Africa, while \textit{P. vivax} infection happens more in South America, compared to \textit{P. falciparum}. Meanwhile, in South-eastern Asia and Western Pacific, both \textit{P. falciparum} and \textit{P. vivax} are prevalent. Even though \textit{P. malariae} could arise in all malarious areas, the \textit{P. malariae} prevalence is usually low. Meanwhile in tropical Africa, \textit{P. ovale} is prevalent and sometimes \textit{P. falciparum} and \textit{P. malariae} co-infection is found (Autino et al., 2012). However, in the recent years, the fifth human malaria parasite, known as \textit{P. knowlesi} that was formerly infecting monkeys has been discovered, raising concerns on zoonotic transmission (White, 2008). \textit{P. knowlesi} infection occurs only in forested area in certain countries in South-East Asia (Autino et al., 2012). The zoonotic malaria species \textit{P. knowlesi} has become the main cause of human malaria in Malaysian Borneo (Fornace et al., 2016; William et al., 2011; Yusof et al., 2016).

The clinical features shown on malaria patients usually appear during parasite's maturation in human blood and the symptoms emergence coincides with the release of antigens during the host cell rupture (Trampuz et al., 2003). According to its severity, malaria is usually classified as uncomplicated (or mild) or complicated (severe) (Phillips et al., 2017). To circumvent severe illness or fatality, an appropriate management of malaria is vital. At present, malaria treatment according to the standard treatment recommended by the WHO, is Artemisinin Combination Therapy (ACT) as the first line of malaria treatment for countries that are prevalent with \textit{P. falciparum} infection. In areas of multidrug resistance (South-East Asia), drugs presently used for malaria treatment including combination of artemether-lumefantrine or artesunate + mefloquine while in Africa artemether-lumefantrine, artesunate + amodiaquine, or artesunate + sulfadoxine-pyrimethamine is used. The second line treatment could be alternative of ACT, quinine + tetracycline or doxycycline or clindamycin. Chloroquine is generally used for \textit{P. vivax}, \textit{P. ovale} and \textit{P. malariae}. (CDC, 2015; WHO, 2015).

The reduction of death due malaria nowadays is largely contributed by the collective efforts of adoption of new-artemisinin-based medicine, increased of investments for research and development that leads to improvements in antimalarial treatments. Jagoe mentioned in the Medicines for Malaria Ventures (MMV) that a single dose treatment has been introduced in 2018 for vivax malaria. The current treatment always lead to poor compliance because of the treatment period; this could replace the current 14 days treatment to stop relapse (Jagoe, 2018). The trial for the new vivax malaria treatment was performed in Brazil, Cambodia, Ethiopia Peru, Philippines and Thailand, where they have selected 522 malaria patients with confirmed infection with \textit{P. vivax} and normal glucose-6-phosphate dehydrogenase (G6PD), resulted in significantly lower risk of recurrence of \textit{P. vivax} as compared to placebo in patient with normal G6PD activity (Lacerda et al., 2019).

Many approaches using the latest technology has been conducted to develop new drug candidates for the treatment of antimalarials, as alternative to the currently available treatments. Four years ago, a team at MVV lead by Burrows and colleagues has established an outline for malaria drug design by defining the minimum acceptable profile of target candidates and target product for developing a novel malaria therapy. Target candidate profile (TCP) with their mechanism of action has been listed out in their review. Each TCP has their own mechanism of actions, as for example TCP1 targeted molecules that would clear asexual plasmodial blood stages in patients, while TCP3 aim the molecule that aids in clearing the dormant hypnozoites as prevention of relapses due to \textit{P. vivax} or \textit{P. ovale} infections. Meanwhile, TCP4 and TCP5 have different target where TCP4 targeted molecules that can clear the liver stage infection. However, TCP5 and TCP6 action by blocking the transmission, TCP5 rendering the gametocyte non-functional and TCP6 kill the mosquito following a blood meal (Burrows et al., 2017). MMV predicted that only less than two percent of project for new antimalarial will proceed to final stage of clinical trial.

Okombo and Chibale reviewed in 2018 on the latest progress of drug candidates as described in MMV and the NIH-hosted repository for clinical trials. A literature search was performed by Okombo and Chibale from articles in the life sciences journal archive and PubMed that was published in the last five to six years, where it was described that the drug candidate for antimalaria at present are under various phases of
consideration for development. Six drugs were at lead optimization stage, five drugs are at preclinical stage, three antimalarial drugs candidates are already for clinical evaluation and additional six drug candidates are in product development under patient exploratory stage. These current discoveries will require long term investments, scientific venture and political support to warrant the decrease of disease burden globally (Okombo & Chibale, 2018).

1. Antimalarial resistance

Over the years, the efficacy of most antimalarial drugs is declining, which is mainly due to the more widespread resistance. This issue is further exacerbated by the fact that the compounds currently used in malaria treatment share related mechanisms of action. This hinders the efforts to reduce the morbidity and mortality rates related to this disease (Olliaro & Mussano, 2003). Resistance of P. falciparum towards antimalarial starting from the first report of quinine resistance in 1910, followed by chloroquine in 1960 (Blount 1967; da Silva & Benchimol, 2014; Tan et al., 2014). An analysis of molecular, genetic and biochemical approaches to P. falciparum gene resistance towards chloroquine has been conducted by Le Bras & Durand (2003), which the study aimed to elucidate P. falciparum multidrug resistance 1 gene (pfmdr1). The authors identified a gene on chromosome 5, encoding a P-glycoprotein homolog 1 and P. falciparum chloroquine resistance transporter (pfcr) on chromosome 7 as the main determinants of chloroquine resistance. Their findings have also shown that mutations in pfmdr1 and pfcr genes eventually lead to chloroquine resistance. Mutations in these genes causes reduction in chloroquine uptake by the parasite’s vacuole (Le Bras & Durand, 2003; Ibraheem et al., 2014). Chloroquine resistance of P. vivax has already grasped a worrying prevalence in Indonesia, East Timor and Papua New Guinea. Recently, chloroquine resistance of P. vivax infections in Guyana, Peru and Brazil has been investigated in in vivo studies (Gonçalves et al., 2014). In the same year, molecular evidence of increased resistance to chloroquine and sulphadoxine/pyrimethamine has been reported. Both in vivo treatment efficacy and molecular assays were used to identify the range of P. falciparum resistance towards both drugs in two of north-east Indian state. The study reported 81.5% treatment failure for chloroquine and 13.7% treatment failure to sulphadoxine / pyrimethamine. Moreover, 99% of the failure had mutant pfcr genotype (76T), while 68% had mutant of pfmdr-1 genotype (86Y). The study indicates high level of resistance was observed in North-east India and it triggered an alarm for malaria management in north-east India (Mohapatra et al., 2014). Another study conducted during 2008–2013 in Kolkata and Purulia, India, resulted in a rise of in vitro chloroquine resistance to P. falciparum, even after five years following chloroquine withdrawal (Das et al., 2017).

Since chloroquine is no longer used in treating P. falciparum infection, a standardized treatment using ACT has been employed to replaced chloroquine. Presently, ACT is still the most effective treatment for malaria since to date, no other alternative treatment can match its potency (WHO, 2015). However, in an earlier study, Rogers et al. (2009) found that artesunate-mefloquine, a type of ACT antimalarial, has started to fail in southern Cambodia (Rogers et al., 2009). P. falciparum resistance to artesiminin has also been identified in the Greater Mekong Subregion including Cambodia, Myanmar, Laos, Thailand and Vietnam (WHO 2018). The mutations in the kelch propeller (K13-propeller) domain has been associated with the development of artesiminin resistance involving point mutations at Y493H, C580Y, M476I, R539T and I543T (Ariey et al., 2014; Straimer et al., 2015). The development of P. falciparum resistance towards artesiminin derivatives is causing limited efficacy of the current antimalarial drugs (Ringwald et al., 2012). An investigation into the efficacy and safety of artesiminin-based antimalarials in the treatment of uncomplicated malaria for children in southern Tanzania indicated that artemether-lumeferantrine (AL) is very efficacious in areas of high sulphadoxine–pyrimethamine (SP) resistance (Kabanywanji et al., 2007). More recently, Hastings et al. (2014) reported that artemisinin component typically shows insignificant contribution (<0.0001%) to the therapeutic capacity of the most widely used ACTs (Hastings & Hodel, 2014). On the other hand, a study to determine the efficacy of conventional antimalarials against P. knowlesi, the fifth human malaria parasite, revealed that it is uniformly
highly sensitive to artemisinin, variably and moderately sensitive to chloroquine, and less sensitive to mefloquine (Fatih et al., 2013). Table 1 summarized the regions where antimalaria drug resistance have been reported with respective molecular markers.

Thus, there is a vital need to identify novel targets that can be employed in the improvement of the next generation of antimalarial drugs. Since antimalarial drugs are a vital component of disease control and elimination, potential failure of these drugs in the future would hinder the efforts dedicated to disease eradication and treatment. The aim of this review is to elucidate the applicability of enzymes found in *Plasmodium spp.* as possible therapeutic targets in combating malaria. In order to achieve this objective, pertinent information related to antimalarial targets, especially the enzymes involved in specific pathways, the mechanism of action of the enzymes and inhibitors, as well as the strategies used to discover new antimalarial drugs are discussed and presented in the following sections.

**Table 1.** Specific regions where antimalaria drug resistance have been reported with their respective molecular markers

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Species</th>
<th>Region</th>
<th>Molecular marker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td><em>P. falciparum</em></td>
<td>Indonesia, East Timor, Papua New Guinea, Guyana, Peru and Brazil</td>
<td>Mutation on <em>pfmdr1</em> and <em>pfcrt</em> genes</td>
<td>(Le Bras &amp; Durand, 2003)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td><em>P. vivax</em></td>
<td>Indonesia, East Timor, Papua New Guinea Guyana, Peru and Brazil</td>
<td>Mutation on <em>pfmdr1</em> and <em>pfcrt</em> genes</td>
<td>(Gonçalves et al., 2014)</td>
</tr>
<tr>
<td>Chloroquine and sulphadoxine/pyrimethamine</td>
<td><em>P. vivax</em></td>
<td>North-east India</td>
<td>Mutation on <em>pfmdr1</em> and <em>pfcrt</em> genes</td>
<td>(Mohapatra et al., 2014)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td><em>P. falciparum</em></td>
<td>Kolkata and Purulia, India</td>
<td></td>
<td>(Das et al., 2017)</td>
</tr>
<tr>
<td>Artesunate-mefloquine</td>
<td><em>P. falciparum</em></td>
<td>Southern Cambodia</td>
<td></td>
<td>(Rogers et al., 2009)</td>
</tr>
<tr>
<td>Artemisinin</td>
<td><em>P. falciparum</em></td>
<td>Cambodia, Myanmar, Laos, Thailand and Vietnam</td>
<td>Mutations in the kelch propeller (K13-propeller)</td>
<td>(WHO, 2018)</td>
</tr>
<tr>
<td>Sulphadoxine–pyrimethamine</td>
<td><em>P. falciparum</em></td>
<td>Tanzania</td>
<td></td>
<td>(Kabanywanyi et al, 2007)</td>
</tr>
</tbody>
</table>

**2. Antimalarial target**

Over the years, most of the antimalarial drug discovery efforts tend to focus on parasite-specific processes, such as hemoglobin degradation, parasite’s egress from the host cell, and host cell invasion by the parasite. As these approaches are becoming increasingly ineffective in combatting and treating malaria, it is essential to conduct further studies aiming to identify new targets for the development of antimalarial drugs. The findings are expected to facilitate construction of new compounds that can interact with the target receptor to diminish the malaria parasite. Targets such as mitochondrion, apicoplast and cytoplasm of *Plasmodium* have been studied extensively in extant studies and their potential as drug targets has been elucidated. Table 2 summarizes the Plasmodial enzymes in metabolic pathways that have been identified as drug targets.
Table 2. Plasmodial enzymes in metabolic pathways as therapeutic targets.

<table>
<thead>
<tr>
<th>Metabolic pathway</th>
<th>Inhibitor</th>
<th>Therapeutic target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron and heme metabolism</td>
<td>Artemisinin and its derivatives</td>
<td>Pf ring stage activates ART</td>
<td>(Klonis, Creek, &amp; Tilley, 2013)</td>
</tr>
<tr>
<td>Small molecule species specific inhibitors of Plasmodium falciparum (DHOD)</td>
<td>Dihydroorotate dehydrogenase</td>
<td></td>
<td>(Patel et al., 2008)</td>
</tr>
<tr>
<td>Triazolopyrimidine-based</td>
<td>Dihydroorotate dehydrogenase</td>
<td></td>
<td>(Gujjar et al., 2009)</td>
</tr>
<tr>
<td>Aryl and aralkyl amine-based triazolopyrimidine (SPROUT-designed inhibitors)</td>
<td>Dihydroorotate dehydrogenase</td>
<td></td>
<td>(Gujjar et al., 2011)</td>
</tr>
<tr>
<td>Pyrimidine biosynthesis pathway</td>
<td>Tetracyclic benzothiazepines 4(1H)-pyridones</td>
<td>Cytochrome bc1</td>
<td>(Dong et al., 2011)</td>
</tr>
<tr>
<td>Mitochondrial inner membrane enzyme</td>
<td>Heterocyclic Quinolones</td>
<td>NADH: ubiquinone oxidoreductase</td>
<td>(Leung et al., 2012)</td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>Antifolates</td>
<td>Dihydrofolate reductase and dihydropteroate synthase</td>
<td>(Hyde et al., 2005)</td>
</tr>
<tr>
<td>Folate pathway</td>
<td>Sulfur based drugs (analogs of sulphanilamide)</td>
<td>Dihydropteroate reductase and dihydropteroate synthase</td>
<td>(Patel et al., 2006)</td>
</tr>
<tr>
<td>Quinazolinones (GTP analogue inhibitor (8-oxo-GTP))</td>
<td>Dihydrofolate reductase</td>
<td></td>
<td>(Patel et al., 2017)</td>
</tr>
<tr>
<td>GTP cyclohydrolase I</td>
<td>GTP cyclohydrolase I</td>
<td></td>
<td>(Kümpornsin et al., 2014)</td>
</tr>
<tr>
<td>Galloflavin</td>
<td>Lactate dehydrogenase</td>
<td></td>
<td>(Manerba et al., 2012)</td>
</tr>
<tr>
<td>Itraconazole, atorvastatin and posaconazole</td>
<td>Lactate dehydrogenase</td>
<td></td>
<td>(Penna-Coutinho et al., 2011)</td>
</tr>
<tr>
<td>Azole-based compound</td>
<td>Lactate dehydrogenase</td>
<td></td>
<td>(Cameron et al., 2004)</td>
</tr>
<tr>
<td>3-Br-isoxazoline</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td></td>
<td>(Bruno et al., 2016)</td>
</tr>
<tr>
<td>Analogues of inorganic diphosphate</td>
<td>T. cruzi hexokinase</td>
<td></td>
<td>(Hudock et al., 2005)</td>
</tr>
<tr>
<td>PFK inhibitors with antiparasitic activity</td>
<td>hexokinase</td>
<td></td>
<td>(Davis et al., 2016)</td>
</tr>
</tbody>
</table>

2.1 The mitochondrion target

According to Goodman et al., 2017, Plasmodium mitochondrion is an effective drug target with safe effective drugs for medical use. The mitochondrion is an organelle that plays a key role in the eukaryotic cell energy production. Plasmodium mitochondrion, a single tubular organelle structure (6-kb mtDNA) is highly fragmented rRNA gen and only encodes three genes for proteins (Das et al., 1997; Hikosaka et al., 2011). Plasmodium and the host’s mitochondrion are different in terms of molecular and their function. Plasmodial mitochondrial is essential in the parasite’s life cycle (Lunev et al., 2016), where mitochondrial dysfunctional can cause cell death. An overview of studies examining mitochondria
of *Plasmodium* as a drug target conducted by Hikosaka *et al.* (2015) indicated that the enzymes of mitochondrial tricarboxylic acid (TCA) cycle and mitochondrial electron transport chain (mtETC) are attractive drug targets. TCA cycle, also known as Krebs cycle, is one of the important stages in aerobic respiration. It is well-established that the substrate for TCA cycle is acetyl-CoA from pyruvate oxidation or fatty acid/protein degradation. Lunev *et al.* (2016) also has discussed in detail regarding the potential pathways of the mitochondrial and carbon metabolisms, such as pyrimidine biosynthesis, aspartate metabolism and mitochondrial tricarboxylic acid (TCA) cycle as drug targets in *P. falciparum* (Lunev *et al.*, 2016). Although *P. falciparum* possesses all enzymes of the TCA cycle, it has been suggested that at least the asexual stages do not require TCA cycle for energy generation (van Dooren, Stimmmer and McFadden 2006) and rely on glycolysis and fermentation for energy (Vander Jagt *et al.*, 1990). Moreover, Hikosaka *et al.* (2015) cautioned that drug targeting TCA cycle and mtRTC was a difficult approach, as it required rigorous biochemical analysis due to the complexity associated with obtaining intact and pure mitochondria from the parasites. Furthermore, studies mentioned that during erythrocytic cycle of plasmodium, oxidative phosphorylation is not crucial for the *Plasmodium* as the parasites depend mainly on glycolysis for the source of energy to survive during the blood stage (Ke *et al.*, 2015; MacRae *et al.*, 2015; Bryant *et al.*, 1964; Roth *et al.*, 1988). Thus, Lunev *et al.* (2016) generally believed that the role of mitochondria in the parasite during blood stage is mainly for the maintenance of inner mitochondrial potential. Hence, designing antimalarial drugs by targeting the mitochondrion, specifically by failing the inner mitochondrial potential and inhibit *Plasmodium* growth is still relevant, as it may potentially deliver new drugs for malaria treatment.

Nevertheless, mitochondrial electron transport remains as target for the existing and new antimalarials. An analysis has suggested that a mixture of chloroquine or its analogue, together with another drug, inhibits carbon fixation and/or increases oxidative stress, where this should increase the clearance of *P. falciparum* from the host system (Tewari *et al.*, 2017). Compounds such as atovaquone are known to target the mitochondrial electron transport chain, since it blocks the flux of metabolites through the TCA cycle (Ke *et al.*, 2015). The details of the studies of enzymes in these pathways are tabulated in Table 2.

Even though malaria parasite’s mitochondria metabolic processes are limited, and less pathways appear during the intraerythrocytic cycle for the parasite survival, it is encouraging that various number of compounds selectively aiming *Plasmodium* mitochondrion. Inhibition of mitochondria resulting in accumulation of free fatty acid and triglycerides in the cytoplasm and their derivatives, which have toxic effects potential on mitochondrial respiration, ketone body production, gluconeogenesis and ATP synthesis (Olszewska & Szewczyk, 2013).

### 2.2 Apicoplast target

Apicoplast (vestigial plastid) is a chloroplast-like organelle characterized by a unique non-photosynthetic plastid. Apicoplast can be found in most parasites of Phylum Apicomplexa, a name derived from apical complex, except in *Cryptosporidium* spp. (Köhler *et al.*, 1997; McFadden *et al.*, 1996). Apical complex is an anterior structure that allows the parasite to invade the host cells and establish themselves in the host. Apicoplast is a specialized organ located below apical complex that performs multiple functions but its capacity to photosynthesize is limited. In addition, it holds a wide range of metabolic pathways that can be used by the parasite. Some of the product components are critical for the host cell invasion (Ralph *et al.*, 2001; Mukherjee & Sadhukhan, 2016). However, apicoplast retains the typical plastid functions, such as isoprenoid, fatty acid, and haem syntheses (Table 1), while also carrying cellular processes, such as deoxyribonucleic acid (DNA) replication, transcription, translation, and post-translational modification (Mukherjee & Sadhukhan, 2016). Nonetheless, the reason why apicoplast is essential to the parasite’s survival is still unknown (Lim & McFadden, 2010).

Apicoplast as an antimalarial drug target has been examined by several researchers (Botté *et al.*, 2012; Mukherjee and Sadhukhan 2016; Ralph, D’Oombrain, & McFadden, 2001). According to their findings, the apicoplast target for antimalarial is based on the metabolic activity in
the *Plasmodium* itself. As reviewed by Mukherjee & Sadhukhan (2016), in apicoplast DNA replication, gyrase A (GyrA) and gyrase B (GyrB) play an important role in *Plasmodium*, where DNA cleavage and wrapping domains were located in GyrA subunit, whereas adenosine triphophosphate (ATPase) and DNA binding/GyrA interaction domains are present in the GyrB subunit, where this structure exhibit strong homology to *Escherichia coli*. In *E. coli*, aminocoumarin drug novobiocin inhibits the ATPase activity of GyrB, while in *P. falciparum* the drug was shown to competitively inhibit the apicoplast’s ATPase activity and DNA replication, which causing a lag in conversion of the parasite’s stages from trophozoite to schizont stage. Novobiocin also disrupt the activity of *P. falciparum* GyrB ATPase that causes the parasite’s death in cultures. On the other hand, the review also mentioned ciprofloxacin, which shown to inhibit apicoplast DNA replication in *P. falciparum* (Mukherjee & Sadhukhan, 2016).

In a more recent study, Rai et al. (2017) noted that understanding of the function of apicoplasts will benefit in designing better therapeutics through calcium signaling because it plays a vital role in the development and growth of *Plasmodium*, specifically in apicoplast transcriptional regulation (Cheemadan et al., 2014; M. Rai et al., 2017). Cheemadan et al. (2014) studied the role of calcium signaling in the transcriptional regulation of the apicoplast genome of *P. falciparum*. In order to assess the effect of changing calcium distribution within the cell, the transcriptional responses of *P. falciparum* to two calcium ionophores was analysed. It was found that both inhibitors induced overlapping but not identical changes of the *P. falciparum* transcriptome. However, both inhibitors caused strong inhibition of transcriptional activity of all the important genes in the apicoplast genome. Due to this situation, the authors identified MAL13P1.156, which is a nuclear encoded apicoplast targeted protein that transports a calcium binding domain. Overexpression of the protein causing resistance of *P. falciparum* towards ionomycin and it was proposed that this protein possibly play an important role in calcium dependent signaling pathways in apicoplast (Cheemadan et al., 2014).

In addition, apical membrane antigen 1 (AMA1) is one of the components in the invasion machinery of *Plasmodium*. AMA1 was found to be an important candidate for malaria vaccine. A review beheld at both structural details and functional significance of interactions at the hydrophobic cleft of AMA1 have been elucidated and it is suggested as an ideal target for the development of drugs that can prevent host cell invasion by malaria parasites (Macraird et al., 2011).

### 2.3 Cytoplasmic target

An apicomplexan including *Plasmodium spp.*, *Toxoplasma gondii*, and *Cryptosporidium parvum*, as well as opportunistic pathogens of immunocompromised individuals *Eimeria spp.* and *Theileria spp.*, share distinct morphological features and cytoskeletal organization. Cytoskeletal system is multifaceted, comprising of interlinking filaments and tubules that spread throughout the cytoplasm. In *Plasmodium*, various metabolic pathways can be found in the cytoplasm, such as, folate metabolism and glycolysis. These metabolic pathways consist of hundreds of enzymes that may be potential drug targets (Gardner et al., 2002). A general overview adapted and modified from Gardner et al., 2002 on these pathways is shown in Figure 1.

### 2.4 Enzymes involved in folate pathway

Empirical evidence also indicates that folate pathway is vital for synthesizing amino acids, as it is important for building of proteins for the malaria parasites survival. By referring to previous literatures regarding folate pathway, several drugs were developed to disrupt folate pathway with the aim of developing new treatment and prophylaxis for malaria. Folate pathway is also critical in providing cofactors for the metabolic events. The enzymes in this pathway serve as molecular targets, including dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). DHFR aids in the oxidation of tetrahydrofolate (THF) molecule to DHF and its reconversion to the THF form (Hyde, 2005). DHPS is an enzyme with two binding pockets, one of which binds to dihydropterin pyrophosphate (DHPP), while the other binds with p-amino benzoic acid (pABA). DHPS catalyzes the reaction that produces 7,8-dihydropteroate from these two substrates.
Among the antimalarial drugs presently in use, the antifolates have emerged as the most promising owing to their well-defined molecular targets—dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS)—that are also involved in the folate metabolic pathway. Antifolates are inhibitors of folate synthesis or folate conversions and are currently used for malaria treatment. These are first-line drugs in some of the African countries, as they are a cost-effective choice for fighting chloroquine-resistant malaria (Hyde, 2005). The antifolates have also been developed as synergistic combinations of DHFR and DHPS inhibitors. Antifolates, such as sulfur-based drugs, which are analogs of sulfanilamide, can inhibit DHPS. These compounds compete with para-aminobenzoic acid in the DHPS active site. However, due to their inherent toxicity, there is paucity of studies on the sulfur-based drugs, which limits their use in antimalarial treatment (Nzila, 2006). Another evaluation of antimalarial screening of quinazolinones was carried out by Patel et al., (2017) both computationally as well as in vitro and its findings revealed their DHFR inhibitory potency. The main contribution of this study stems from identifying five out of nineteen potent antimalarial molecules (Patel et al., 2017).

Other enzymes in folate pathways were defined in an earlier work by Lee et al. (2001), which described the gene that encodes GTP cyclohydrolase I (gtp-ch), catalyzing the conversion of GTP to dihydronopterin via triphosphate. Polymerase chain reactions and DNA sequencing were performed to isolate the gtp-ch gene of P. falciparum. The gene was identified by using six primers and direct sequencing of this fragment discovered an open reading frame that are comparable with the predicted protein sequence and those of known gtp-ch molecules. This gene is attractive for novel antimalarial therapeutic target because it is expressed in blood-stage parasites and gtp-ch provides the rate-limiting steps in folate pathway, as shown in another microorganism (Lee et al., 2001). Years later, Kümpornsin et al. (2014) studied the enzymatic activity and genetic
complementation for \textit{P. falciparum} GTP cyclohydrolase I (\textit{PfGCH1}). Its findings indicated that this could be a new approach to antimalarial drug development, since the assay of this enzyme showed an inhibitory effect by 8-oxo-GTP, a known GTP analogue inhibitor (Kümpornsin \textit{et al.}, 2014).

### 2.5 Enzymes involved in glycolytic pathway

Finding the alternative molecular targets for antimalarial drug design, specifically on the energy-generating pathway of carbohydrate metabolism is deemed essential in most drug design studies. Glycolysis, the metabolic pathway for carbohydrate metabolism, is an ancient, conserved metabolic energy-producing machinery that converts glucose to pyruvate and lactate under aerobic and anaerobic conditions, respectively. The enzymes in this pathway are crucial for parasite survival and growth. It is well-established that \textit{P. falciparum} solely depends on glycolysis for energy generation and meets its energy needs by anaerobic metabolism of glucose to lactate (Sabbatani, Fiorino, & Manfredi, 2010). Glycolysis occurs in two distinct phases—the preparatory and the payoff phase. The preparatory phase is the glucose activation phase, where phosphorylation of glucose and its conversion to glyceraldehyde-3-phosphate takes place, while the payoff phase is the extraction stage, during which conversion of glyceraldehyde-3-phosphate to pyruvate, as well as the coupled formation of ATP, occurs (Giri, 2016). Ten enzymes are involved in glycolysis, namely hexokinase, phosphohexose isomerase, phosphofructokinase-1, aldolase, triose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase. During anaerobic condition, an additional enzyme—lactate dehydrogenase—is required. Genome sequence analysis by Gardner \textit{et al.} (2002) has provided a comprehensive understanding of the metabolic potential of \textit{P. falciparum}.

Glycolysis is the key metabolic pathway for ATP production in malaria parasite and is pivotal for its survival during the \textit{Plasmodium} intra-erythrocytic cycle. The glycolysis rate in \textit{P. falciparum}-infected erythrocytes is 20–100 times higher than in uninfected erythrocytes, which renders them valuable indicators of current infections (Iqbal \textit{et al.}, 2004; Singh & Daneshvar, 2013). In a much earlier study, Roth Jr. \textit{et al.} (1988) demonstrated that nearly all glycolytic enzymes were upregulated in \textit{P. falciparum}-infected red blood cells (RBC), which was proportional to the parasitemia level. More recently, Kantele & Jokiranta (2011) revealed that hexokinase, aldolase, enolase, pyruvate kinase, and adenosine deaminase were the most markedly upregulated enzymes. The functions of each enzyme in the glycolytic pathway of malaria parasites have been reviewed extensively by Alam \textit{et al.} (2014). The authors also investigated the functions of \textit{P. falciparum} glycolytic enzymes as a part of their review. They noted its exceptional structural differences and functional features, suggesting that these could be exploited as therapeutic targets.

Research on \textit{Plasmodium} lactate dehydrogenase has revealed that \textit{pLDH} could be a diagnostic biomarker, as well as antimalarial inhibitory target (Jain \textit{et al.}, 2014; van Niekert \textit{et al.}, 2016). Manerba \textit{et al.} (2012) conducted their study aiming to verify galloflavin (CAS 568-80-9) as a novel inhibitor of lactate dehydrogenase. The authors reported that, in cultured tumor cells, galloflavin blocked the aerobic glycolysis at micromolar concentrations, but did not affect the cell respiration or induced cell death by triggering apoptosis. Additionally, 50 commercially-available compounds that have been selected through molecular docking approach were tested by Penna-Coutinho \textit{et al.} (2011) against \textit{P. falciparum} lactate dehydrogenase (\textit{PfLDH}) and three compounds (itraconazole, atorvastatin and posaconazole) have been identified with the closest binding energy values to NADH. These were subsequently proven active in immunoenzymatic assays. The study findings further demonstrated that molecular docking research can be a reliable approach for discovering new antimalarial drugs (Penna-Coutinho \textit{et al.}, 2011). Following an earlier study on azole-based compound, which is also an inhibitor of \textit{PfLDH}, Cameron \textit{et al.} (2004) suggested that these compounds have limited chances for additional derivatization due to the close contacts made within the active site of the enzyme. As the authors also noted that they appeared to have limited cellular uptake in the current form, they called for further development...
of extended azole-like compounds, which may result a beneficial route for the improvement of novel antimalarials (Cameron et al., 2004).

Other enzymes in the glycolytic pathway of Plasmodium were also widely studied, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in particular. GAPDH is an important glycolytic enzyme that catalyzes the oxidation of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate (1,3-BPG), coupled to the reduction of nicotinamide adenine dinucleotide (NAD+) to NADH (Bruno et al., 2016). Recent investigations have revealed that this enzyme is promising as a new malaria diagnostic biomarker because native PfGAPDH protein levels were 4–6 times higher than the current target PfLDH (Krause et al., 2017). However, GAPDH has also been explored as potential antimalarial drug target. Compounds based on the 3-Br-isoxazoline scaffold completely inhibit GAPDH from P. falciparum by selectively alkylating all four catalytic cysteines of the tetramer, but only inhibit 25% of human orthologue (hGAPDH) (Bruno et al., 2016).

Hexokinase has also been the subject of extensive research, as it is the first enzyme in the glycolytic pathway. An inhibition study on hexokinase of Trypanosoma cruzi, another species of blood protozoa, by bisphosphonates, showed that these non-hydrolyzable analogues of inorganic diphosphate are potent inhibitors of T. cruzi hexokinase (Hudock et al., 2005). More recently, Davis et al. (2016) attempted to identify the inhibitor for P. falciparum hexokinase, where 57,654 molecules were screened from multiple small-molecule collections. Their findings revealed that the most potent inhibitors (GSK-650394) have 50% inhibitory concentrations as low as ~1 µM, and some were found to have low-micromolar concentrations (NGC 0099116 and NCGC0009290) that were 50% effective against asexual blood stage of P. falciparum parasites.

3. Approaches employed in the discovery of new antimalarial drugs
3.1 Computational approach
Drug design and discovery pipeline comprises all the drug development stages, from identifying a target to clinical trials for testing the drug on human subjects. Hence, drug discovery is typically considered a time-consuming and costly process. However, computer-aided drug design (CADD), a computational technology aimed at assisting the antimalarial drug discovery process, has become indispensable in recent years. It makes use of the structural knowledge of either the target (structure-based) or known ligands with bioactivity (ligand-based) to facilitate the identification of likely candidate drugs (Macalino et al., 2015).

A study benefiting from this approach was performed by Granchi et al. (2015) with the aim of finding new human lactate dehydrogenase 5 (LDH5) inhibitors. As part of this investigation, an automated docking-based virtual screening platform was developed by considering different protein conformations and the consensus docking strategy. The authors discovered that two of the ten of the selected compounds efficiently inhibit the enzyme activity via enzymatic assays (Granchi et al., 2015). Given that it avoids carrying out enzymatic assays of thousands of compounds, CADD is highly cost- and time-efficient, as it helps optimizing the work in the laboratory. Several studies have been performed using both structure-based drug design (SBDD) and ligand-based drug design (LBDD) approaches in finding new inhibitors for the enzymes in Plasmodium spp. as the target, as summarized in Table 3.

Huthmacher et al. (2010) conducted their study with the goal of elucidating the capability of computational methods to foresee the metabolic activities during different stages of P. falciparum life cycle, which later led to the identification of new drug targets. Their findings suggested that the set of significant enzymes projected by flux balance approach signifies a reliable beginning for further drug development (Huthmacher et al., 2010).

3.2 Natural products
It well-elucidated that countless compounds for drug discovery can be found in natural products. In the past two decades, their use has declined due to the technical difficulties associated with screening natural products in high-throughput assays compared to molecular targets (Harvey et al., 2015). Azas et al., (2002) evaluated potent in vitro synergistic antimalarial interactions between the extracts of Mitragyna enermis (Wild) O. Kuntze, Rubiaceae, Nauclea latifolia (Sm.) Giera senegalensis (GMel), Combretaceae and Feretia apodantera.

Plasmodial enzymes as new antimalarial drugs: a review

(Del) by conducting isobologram analysis. The findings yielded revealed that tetrahydroharman isolated from *G. senegalensis* exhibits antimalarial activity with neither cytotoxicity nor genotoxicity found in Salmonella Ames test, with and without metabolic activation (Azas et al., 2002).

Table 3. Implementation of SBDD and LBDD approaches to find new inhibitors in malaria drug discovery

<table>
<thead>
<tr>
<th>Methods</th>
<th>Species</th>
<th>Enzyme target</th>
<th>Inhibitor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular modeling</td>
<td><em>Plasmodium falciparum</em></td>
<td>Enoyl reductase</td>
<td>VRC-007 and VRC-009 (analogues of N-benzylidene-4-phenyl-1,3-thiazol2-amine)</td>
<td>(Morde et al., 2009)</td>
</tr>
<tr>
<td>Molecular modeling</td>
<td><em>Plasmodium falciparum</em></td>
<td>Dihydroorotate dehydrogenase</td>
<td>KMC-3 and KMC-15 (derivatives of N-phenylbenzamidine)</td>
<td>(Desai, Shaikh, &amp; Coutinho, 2011)</td>
</tr>
<tr>
<td>Molecular docking and 3D-QSAR</td>
<td><em>Plasmodium falciparum</em></td>
<td>Cysteine proteases</td>
<td>Derivatives of peptidyl vinyl sulfone</td>
<td>(Teixeira et al., 2011)</td>
</tr>
<tr>
<td>3D-QSAR, molecular dynamics, docking, and</td>
<td><em>Plasmodium falciparum</em></td>
<td>Dihydroorotate dehydrogenase</td>
<td>Derivatives of triazolopyrimidine</td>
<td>(Shah et al., 2012)</td>
</tr>
<tr>
<td>genetic algorithm-based methods</td>
<td></td>
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<tr>
<td>Modelling the evolution of drug resistance</td>
<td><em>Plasmodium</em></td>
<td>Dihydrofolate reductase</td>
<td></td>
<td>(Hecht &amp; Fogel, 2012)</td>
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<td>in malaria.</td>
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<tr>
<td>Docking and in silico ADMET</td>
<td><em>Plasmodium falciparum</em></td>
<td>S-adenosyl-L-homocysteine</td>
<td>Noraristeromycin, curcumin and its derivatives</td>
<td>(Singh et al., 2013)</td>
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<tr>
<td>Ligand-based 3D-QSAR analysis and virtual</td>
<td><em>Plasmodium falciparum</em></td>
<td>Glutathione reductase</td>
<td>N-[(2,3-dihydrobenzo[b][1,4]dioxin-6-yl]-2-(5-isopropyl-2-methoxyethyl)-4H-1,2,4-triazol-3-ylthio)acetamide</td>
<td>(Kamaria &amp; Kawathekar, 2014)</td>
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<tr>
<td>screening</td>
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<td>Molecular docking</td>
<td><em>Plasmodium falciparum</em></td>
<td>Spermidine synthase</td>
<td>(1R,4R)-(N1-(3-aminopropyl)-trans-cyclohexane-1,4-diamine and analogue N-(3-aminopropyl)-cyclohexylamine</td>
<td>(Burger et al., 2015)</td>
</tr>
<tr>
<td>Molecular docking</td>
<td><em>Plasmodium falciparum</em></td>
<td>Transketolase</td>
<td>6′-Methyl-Thiamin Diphosphate</td>
<td>(Hasan et al., 2015)</td>
</tr>
<tr>
<td>In silico screening</td>
<td><em>Plasmodium falciparum</em></td>
<td>Enoyl-ACP reductase</td>
<td>Pyrimidine diones</td>
<td>(Lindert et al., 2015)</td>
</tr>
<tr>
<td>3D QSAR, pharmacophore and molecular docking</td>
<td><em>Plasmodium falciparum</em></td>
<td>M18 aspartyl aminopeptidase</td>
<td>Derivative of quinine, chloroquine, 8-aminoquinoline and known antimalarial M18AAP inhibitors (AID743024)</td>
<td>(Kumari et al., 2016)</td>
</tr>
</tbody>
</table>
More recently, Upadhyay et al. (2014) reported antimalarial potential of extracts and compounds from the chloroform and n-butanol fractions of Ammannia bacifera roots and methanol extract of Ammannia cocinea (AC). These extracts are commonly used in Chinese and Indian medicine as a therapy for diseases. The compounds were isolated from the extracted and fractionated dried and powdered samples, followed by repeated chromatographic separations of the fractions. The compounds effectiveness was demonstrated in vitro through antiplasmodial activity against P. falciparum NF-5. Extracts from AC showed potency in antiplasmodial activity and nontoxic to Vero cells. The authors thus concluded that the selective antiplasmodial activity from these plants will be advantageous in antimalarial drug development and discovery of safer therapeutics (Upadhyay et al., 2014).

Thiengsusuk & Chaijaroenkul (2013) performed an evaluation of antimalarial activities of Thai traditional medicine which consist of 27 medicinal plants and 5 herbal formulations used against chloroquine-resistant (K1) and chloroquine-sensitive (3D7) P. falciparum clones. Their results indicate that that ethanolic extracts from 19 investigated plants/herbal formulations exhibited promising activity against both K1 and 3D7 clones of P. falciparum, with < 50% survival at the concentration of 50 μg/ml. In addition, eight medicinal plants and two herbal formulations that were included in this investigation showed potent antimalarial activity with median range IC50 values < 10 μg/ml against K1 or 3D7 P. falciparum clone or both (Thiengsusuk & Chaijaroenkul 2013).

Sethiya et al. (2014) screened methanol extract of Evolvulus alsinoides for P. falciparum lactate dehydrogenase enzyme inhibitory activity, reporting that E. alsinoides possesses a compound known as scopoletin that potentially inhibits the PfLDH. E. alsinoides is generally recognized as Shankpushpi and is used in traditional remedy for malarial treatment by some ethnic populations of India (Sethiya et al., 2014).

3.3 High throughput screening

High Throughput Screening (HTS) is a drug-discovery method that is widely used in the pharmaceutical industry. It is an automated process that rapidly assays the biological or biochemical activity of many drug-like compounds. During a typical HTS assay, a collection of chemical composites is carefully chosen and tested against a biological target to evaluate the strength of the related inhibition or activation signal (Malo et al., 2006). In order to successfully identify LDH inhibitors from library of a small molecule compound, two label-free high-throughput assays were planned using a kinetic high-throughput screen (VanderPorten et al., 2013). In addition, examination on antimalarial elements from Berberis thunbergii and Eugenia rigida by automated HTS, using UPLC-MS-ELSD-PDA yielded two triterpenoids, namely z-betulinic acid and β-betulinic acid, that were acknowledged as antimalarial active elements from HTS hits of E. rigida (Zhang et al., 2014).

High throughput screening is not only beneficial in the search for antimalarial drugs but is also indispensable in the discovery of small molecules that can interfere with malaria transmission. Plouffe et al. (2016) developed a serum-free one-step assay for malaria transmission-blocking activity, which allowed them to analyze 13,983 known and new compounds. The authors noted that more than 90% recognized antimalarial drugs do not show activity to the late-stage gametocytes. Another high-throughput matrix screening approach was tested by Mott et al. (2015) on 13,910 approved and investigational drugs, allowing the researchers to identify several synergistic and antagonistic antimalarial drug combinations.

CONCLUSION

Malaria is a global issue, with the ongoing malaria transmission, there is a possibility for drug resistance to occur through increasing introduction of parasites to suboptimal drug levels. The growing resistance of P. falciparum to the established antimalarial drugs highlights the need for developing alternative drug regimens. Thus, identifying compounds that may disrupt the enzymes activities that have high control over the pathway’s flux may lead to the discovery of novel and effective antimalarial drugs.
ACKNOWLEDGEMENTS

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REFERENCES


Jagoe, G. 2018. Malaria progress has levelled but new drugs are due.


Cytogenotoxic effects of cypermethrin on root growth: *Allium sativum* as a model system

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Abstract. Cypermethrin is a pyrethroid pesticide used on agricultural farms in Nigeria to control pests of fruits and vegetables but this is highly toxic to aquatic organisms. To determine toxicity of chemicals, *Allium cepa* is commonly used as an established bioassay however the bulb is used whole. *Allium sativum* in contrast is able to be split into cloves of smaller units hence this research aims to validate the potential of *Allium sativum* as a model plant for genotoxicity assessment. In this study, root growth inhibition test and chromosome aberration assay were used and the effective concentration (EC$_{50}$) of cypermethrin was determined from the root growth curve. Furthermore, the mitotic activities of the root meristem were assessed using light microscopy. Treatment of root meristem of *A. sativum* with various concentrations of cypermethrin (0.25, 0.50, 0.75 and 1.0 mg/ml) revealed a reduction in the root length and EC$_{50}$ of 0.44 mg/ml. Also morphological changes such root wilting, dark spots, tenderness of the clove bases and discoloration of the roots were observed. Cytological studies showed a reduction in mitotic index with increasing cypermethrin concentration. Chromosomal aberrations ranging from abnormal metaphases: c-metaphases, disturbed spindles and vagrant metaphases; to abnormal anaphases: laggard chromosomes, chromosome breaks and multipolarities were also recorded. These aberrations reduced with increased concentration of the pesticide leading to the production of lesser number of dividing cells. These show that cypermethrin is genotoxic to the root meristem and *A. sativum* is a suitable model plant for detecting pyrethroid genotoxicity in plant.

Keywords: *Allium sativum*, cytotoxicity, EC$_{50}$, pesticides, pollution monitoring

INTRODUCTION

Pesticides have immensely contributed to the control of pests on farmlands, thereby increasing crop yields. However, they could also be detrimental to human health and ecosystem being one of the most important risks faced by farmers in the developing countries (Coronado *et al.*, 2004).

Cypermethrin is a synthetic, pyrethroid insecticide that has high insecticidal property. It is commonly used in Nigerian farms to control many pests of fruits and vegetables. It is a fast-acting neurotoxic and non-volatile chemical that is easily degraded in soil and plants. The use of cypermethrin has increased several-fold in recent years due to their low mammalian toxicity and limited persistence in soil as compared to organochlorine insecticides (Saxena *et al.*, 2005). However, these compounds are highly toxic to

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fish (Ullah et al., 2018) and other lower aquatic organisms (Dalhoff, 2018) and their widespread use have led to toxic effects in plants, animals and humans (Datta et al., 2003; Biondi et al., 2015; Renu et al., 2017; Yao et al., 2019). The variation in toxicity among organisms may largely be due to differences in their stereo-chemical structures.

The root tips of many plants have long been used as an indicator for chromosome aberration and bioassay studies owing to the fact that plant chromosomes when treated with mutagens produce responses similar to mammals and other eukaryotes hence a number of plant bioassays have been developed to detect environmental mutagens (Grant, 1994). In vivo cytotoxicity tests, using plant such as Allium cepa, have been found to be as effective as in vitro animal testing (Teixeira et al., 2003; Ribeiro et al., 2016), providing valuable information for human health (Ullah et al., 2018). El-Shahaby et al. (2003) stressed the importance of using the Allium cepa test for detecting genotoxicity and evaluating environmental pollution. The plants possess some advantages over other organisms in certain circumstances; it has large chromosomes, low chromosome number and the root meristem contains a high proportion of cells in mitosis (Leme and Marin-Morales, 2009). However, the whole onion bulb has to be used for study of each mutagen hence the need to find an alternately effective plant for bioassay studies. Allium sativum, the common garlic, belongs to the family Alliaceae and genus Allium, it contains chromosomes of similar size and number as Allium cepa and it is also used as a sensitive test system for the evaluation of environmental pollutants and screening of chemicals with genotoxic effects, especially in plants (Saxena et al., 2009; Hemavathi et al., 2015). Unlike A. cepa, A. sativum has the ability to split into natural cloves, this makes it suitable for laboratory work as the clove roots can easily be subjected to singular treatments as against the whole bulb used in Allium cepa. This study therefore aims to validate the effectiveness of Allium sativum as a plant model for determining the genotoxic effects of pyrethroid pesticides on plants. It is hoped that this will prove an adequate replacement for Allium cepa in bioassay studies.

**MATERIALS AND METHOD**

**Test chemical**

A commercial formulation of cypermethrin (trade name: Cyperkill) with a composition of 1.0 mg/ml of the solution was purchased from the market for the experiment.

**Test organism**

Healthy and equal-sized Allium sativum bulbs were properly evaluated prior to purchase. The dry scales of the bulbs were removed and discoid stem of the bulbs were suspended in water for 4 days to ensure proper growth of the roots.

**Experimental design**

Completely randomized design was adopted for the study. The cypermethrin formulation was made into normal concentration of 0.25 mg/ml, suitable for application in agricultural farms, 0.50 mg/ml, 0.75 mg/ml and 1.0 mg/ml undiluted treatments (I) for the experiment with distilled water (C) as a negative control and five replications each for both the microscopic and macroscopic studies.

**Test procedures**

**Root inhibition test**

The macroscopic assessment involved placing the Allium sativum cloves in containers filled with the treatments at room temperature and this was observed daily for 3 days (Fig 1). Parameters such as root length, colour and number of roots were recorded.

**Chromosome aberration assay**

This was achieved following Saxena et al. (2009), Yekeen and Adeboye, (2013), and Renu et al. (2017). The Allium sativum seedlings were treated over 24 h for the microscopic studies. Microscopic analysis was carried out over a cell cycle (24 h) at 6 h interval. The root tips were harvested and fixed in acetic-ethanol (1:3) for 4 h; they were hydrolyzed in 1 N HCl for 6 mins at 60°C and then analyzed for mitotic irregularities. This was done at the 6th, 12th, 18th and 24th hour. The conventional Feulgen-squash method was used to prepare the slides of the root meristems. The slides were mounted on Olympus compound light microscope and observed under
magnifications of 400 µm. Photomicrographs were taken and the mitotic index and chromosome aberrations in mitotic phases was determined. The mitotic index was obtained as follows:

\[
\text{Mitotic index (MI)} = \left( \frac{\text{Number of cells in mitosis}}{\text{Total number of cells}} \right) \times 100
\]

**Statistical analysis**

The means root lengths and standard errors for different concentrations were calculated. The data obtained for each treated group and control were statistically evaluated by Analysis of Variance and Fisher’s Least Significant Difference (FLSD) test at 5% significant level. The mitotic indices were also analyzed using ANOVA and FLSD. Also the half maximal effective concentration (EC\(_{50}\)) was determined.

**RESULTS**

**Root inhibition test**

Treatment of root meristem of *A. sativum* with various concentrations of cypermethrin revealed a reduction in the root length compare to the control. This suppression of root growth varies with the cypermethrin concentration and was followed by different morphological changes ranging from dark spots (after 24 h) to tenderness of the clove bases and discoloration of the roots (after 48 h) (Figure 1). The roots in 0.50 mg/ml solution were slightly discolored after 72 h exposure with no signs of wilt or decay while the roots in the 0.25 mg/ml solution remained unchanged. Differences in mean root lengths were significant (p≤0.05) between different concentrations as well as with control (Table 1). The mean root length (percent control) was plotted against the pesticide concentration (Figure 2). The EC\(_{50}\) of the pesticide was evaluated from the graph to be at 44% of the concentration.

**Chromosome aberration assay**

Treatments of the *Allium sativum* root meristem with the different concentrations of cypermethrin produced results with lower mitotic index compared to the control (Table 2). Mitotic activities were significantly lower (p≤0.05) in the treatments when compared to the control; this was dose dependent and decreased as the concentration of cypermethrin increased. Dividing cells were higher in the control than the treatments and the number of dividing cells decreased as the concentration of cypermethrin increased. Cypermethrin concentration of 0.25 mg/ml with treatment duration of 6 h produced the lowest mitotic inhibition and highest number of dividing cells while complete mitotic inhibition was recorded for roots exposed to 1.00 mg/ml regardless of the exposure duration. Mitotic index of cells exposed to the cypermethrin for 6 h reduced significantly with increasing concentration of the chemical. The means were separated using the Least Significant Difference (LSD\(_{0.05}\)) and these showed significant differences between treatments as well as with control (Table 2).

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Before exposure</th>
<th>After exposure</th>
<th>Increase in root length</th>
<th>Root length (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.24 ± 0.20(\text{a})</td>
<td>4.66 ± 0.24(\text{d})</td>
<td>1.44±0.04</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>2.80 ± 0.14(\text{a})</td>
<td>3.68 ± 0.15(\text{c})</td>
<td>0.88±0.01</td>
<td>51.1</td>
</tr>
<tr>
<td>0.50</td>
<td>3.04 ± 0.27(\text{a})</td>
<td>3.54 ± 0.16(\text{bc})</td>
<td>0.50±0.11</td>
<td>24.7</td>
</tr>
<tr>
<td>0.75</td>
<td>2.98 ± 0.37(\text{a})</td>
<td>3.16 ± 0.12(\text{b})</td>
<td>0.18±0.25</td>
<td>12.5</td>
</tr>
<tr>
<td>1.00</td>
<td>2.76 ± 0.22(\text{a})</td>
<td>2.76 ± 0.09(\text{a})</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NB: Treatment means with same superscripts are not significantly different at 5%.
Figure 1. *A. sativum* bulbs selected for the experiment (A) with their dry scales intact; (B) with their scales removed; (C) Suspension in water for root growth initiation; (D) after root formation, and before treatment; (E) in the treatment containers (F, G, H) softened roots after exposure to T₁, T₂ and T₃ respectively; (I) dried roots after exposure to T₄; (J) control. Magnification - x1.

Figure 2. Effective Concentration (EC₅₀) of cypermethrin on root meristem of *A. sativum.*
Table 2. Mean number of dividing cells and mitotic index of Allium sativum root tip treated with cypermethrin.

<table>
<thead>
<tr>
<th>Treatment (mg/ml)</th>
<th>Duration (h)</th>
<th>Number of dividing cells</th>
<th>Mitotic index (Mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>106</td>
<td>31.86 ± 1.02²</td>
<td></td>
</tr>
<tr>
<td>T₁ (0.25)</td>
<td>6</td>
<td>86</td>
<td>28.66 ± 0.77³</td>
</tr>
<tr>
<td>T₂ (0.50)</td>
<td>6</td>
<td>60</td>
<td>20.06 ± 0.84b</td>
</tr>
<tr>
<td>T₃ (0.75)</td>
<td>6</td>
<td>39</td>
<td>13.12 ± 0.56a</td>
</tr>
<tr>
<td>T₄ (1.00)</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T₁ (0.25)</td>
<td>12</td>
<td>81</td>
<td>26.88 ± 0.38c</td>
</tr>
<tr>
<td>T₂ (0.50)</td>
<td>12</td>
<td>55</td>
<td>18.34 ± 0.92b</td>
</tr>
<tr>
<td>T₃ (0.75)</td>
<td>12</td>
<td>32</td>
<td>10.66 ± 0.72a</td>
</tr>
<tr>
<td>T₄ (1.00)</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T₁ (0.25)</td>
<td>18</td>
<td>76</td>
<td>25.26 ± 0.80c</td>
</tr>
<tr>
<td>T₂ (0.50)</td>
<td>18</td>
<td>50</td>
<td>16.60 ± 0.93b</td>
</tr>
<tr>
<td>T₃ (0.75)</td>
<td>18</td>
<td>32</td>
<td>7.54 ± 0.76a</td>
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<td>T₄ (1.00)</td>
<td>18</td>
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<td>0</td>
</tr>
<tr>
<td>T₁ (0.25)</td>
<td>24</td>
<td>70</td>
<td>23.26 ± 1.39e</td>
</tr>
<tr>
<td>T₂ (0.50)</td>
<td>24</td>
<td>43</td>
<td>14.48 ± 1.07b</td>
</tr>
<tr>
<td>T₃ (0.75)</td>
<td>24</td>
<td>11</td>
<td>3.68 ± 0.37a</td>
</tr>
<tr>
<td>T₄ (1.00)</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Mitotic index was scored in a total of 300 cells.
NB: Treatment means with same superscripts are not significantly different at 5%.

Table 3. Number of chromosomal aberrations observed in A. sativum roots treated with cypermethrin.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Duration (h)</th>
<th>MA</th>
<th>CB</th>
<th>LC</th>
<th>DS/c-M</th>
<th>Vm</th>
<th>Cbr</th>
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<td>T₁</td>
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<td>0</td>
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<td>T₁</td>
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</tbody>
</table>

MA=Multipolar Anaphase; CB=Chromosome bridge; LC=Laggard Chromosome; DS/c-M=Disturbed Spindle c-Metaphase; VM=Vagrant Metaphase; CBr=Chromosome breaks.

The chromosomes were observed at prophase, metaphase, anaphase and telophase stages (Figure 3). Different kinds of chromosomal aberrations were found in the cells treated with cypermethrin however, the number of aberrations did not increase with increasing cypermethrin concentration (Table 3). The most frequent aberrations observed were related to the metaphase and anaphase stages. These were vagrant metaphases, chromosome breaks, disturbed spindles and c-metaphases; chromosome laggards, vagrant quadruple anaphases, triporal anaphases and chromosome bridges (Figure 4).

There was significant difference only in telophase index after 6 h, only prophase index after 12 h but no significant difference in prophase, metaphase, anaphase and telophase indices after 18 and 24 h of treatment period (Table 4).

Cytogenetic effects of cypermethrin on root growth

Figure 3. Normal mitotic stages observed in *A. sativum* treated with cypermethrin. (A) Normal prophase; (B) Normal metaphase with the chromosomes aligned at the plate; (C) Normal early anaphase - chromosomes are migrating towards the poles; (D) Normal late anaphase - chromosomes have reached the poles; (E) Normal telophase.

Figure 4. Aberrant mitotic stages observed in *A. sativum* treated with cypermethrin. A-D: Abnormal metaphase with; (A) c-metaphase: unorganized chromosomes, (B) disturbed spindle, (C) vagrant chromosomes, (D) chromosome break. E-H: Abnormal anaphase with: (E) chromosome bridge, (F) multipolar anaphase with vagrant chromosome, (G) multipolar anaphase - quadruple anaphase, (H) laggard chromosome.

Table 4. Phase index of *A. sativum* root treated with cypermethrin.

<table>
<thead>
<tr>
<th>Duration (h)</th>
<th>Treatment</th>
<th>Prophase</th>
<th>Metaphase</th>
<th>Anaphase</th>
<th>Telophase</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Control</td>
<td>27.66 ± 1.62</td>
<td>24.56 ± 1.66</td>
<td>22.06 ± 3.84</td>
<td>25.76 ± 3.23</td>
</tr>
<tr>
<td>6</td>
<td>T1</td>
<td>29.32 ± 2.19</td>
<td>31.50 ± 2.03</td>
<td>27.98 ± 1.14</td>
<td>11.20 ± 2.22</td>
</tr>
<tr>
<td>6</td>
<td>T2</td>
<td>30.22 ± 3.28</td>
<td>28.56 ± 2.17</td>
<td>22.38 ± 1.75</td>
<td>18.76 ± 3.84</td>
</tr>
<tr>
<td>6</td>
<td>T3</td>
<td>26.60 ± 2.21</td>
<td>25.96 ± 1.08</td>
<td>21.30 ± 1.33</td>
<td>24.92 ± 4.03</td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>0.18 ± 2.46</td>
<td>27.44 ± 2.00</td>
<td>20.62 ± 1.96</td>
<td>21.72 ± 1.70</td>
</tr>
<tr>
<td>12</td>
<td>T1</td>
<td>23.06 ± 1.49</td>
<td>24.64 ± 3.49</td>
<td>26.10 ± 2.25</td>
<td>26.30 ± 3.19</td>
</tr>
<tr>
<td>12</td>
<td>T2</td>
<td>30.74 ± 2.35</td>
<td>26.10 ± 2.99</td>
<td>20.58 ± 2.38</td>
<td>25.32 ± 4.97</td>
</tr>
<tr>
<td>12</td>
<td>T3</td>
<td>22.44 ± 2.55</td>
<td>26.82 ± 2.51</td>
<td>22.28 ± 3.16</td>
<td>28.38 ± 4.69</td>
</tr>
<tr>
<td>18</td>
<td>Control</td>
<td>27.30 ± 3.29</td>
<td>30.48 ± 1.86</td>
<td>22.44 ± 2.46</td>
<td>19.54 ± 1.76</td>
</tr>
<tr>
<td>18</td>
<td>T1</td>
<td>28.22 ± 1.87</td>
<td>28.30 ± 3.14</td>
<td>23.50 ± 2.25</td>
<td>19.84 ± 3.46</td>
</tr>
<tr>
<td>18</td>
<td>T2</td>
<td>32.88 ± 4.65</td>
<td>26.90 ± 3.18</td>
<td>23.86 ± 3.37</td>
<td>15.82 ± 3.72</td>
</tr>
<tr>
<td>18</td>
<td>T3</td>
<td>21.66 ± 3.25</td>
<td>23.66 ± 2.62</td>
<td>29.04 ± 4.84</td>
<td>25.42 ± 5.64</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>29.92 ± 2.71</td>
<td>25.16 ± 1.36</td>
<td>23.44 ± 3.10</td>
<td>21.42 ± 2.68</td>
</tr>
<tr>
<td>24</td>
<td>T1</td>
<td>26.42 ± 2.36</td>
<td>25.48 ± 1.87</td>
<td>23.18 ± 2.21</td>
<td>25.96 ± 1.99</td>
</tr>
<tr>
<td>24</td>
<td>T2</td>
<td>26.88 ± 2.78</td>
<td>26.44 ± 3.91</td>
<td>21.32 ± 2.77</td>
<td>25.96 ± 3.92</td>
</tr>
<tr>
<td>24</td>
<td>T3</td>
<td>21.26 ± 4.92</td>
<td>21.86 ± 5.26</td>
<td>34.82 ± 7.08</td>
<td>22.66 ± 1.58</td>
</tr>
</tbody>
</table>

NB: Treatment means with same superscripts are not significantly different at 5%.
DISCUSSION

Results from this study provide evidence that cypermethrin inhibited root growth and caused root wilt at higher concentrations. The inhibition of growth, according to Grover and Tejpar (1981), may be due to high rate of chemical oxygen demand which affected certain physiological processes leading to the disturbance in the balance between promotors and inhibitors of endogenous growth regulator. There was marked decrease in root length after treatment when compared to the control; this according to Odeigh et al. (1997) is an indication of cytotoxicity. The EC_{50} was achieved at 0.44 mg/ml (44%). This EC_{50} value is greater than the 0.24 mg/ml reported by Saxena et al. (2005) and 0.28 mg/ml recorded by Yekeen et al. (2013) in their study of the effects of cypermethrin on A. cepa. This indicates that A. sativum is more sensitive than A. cepa to cypermethrin and corroborates the findings of Saxena et al. (2008).

The chromosome aberration assay revealed clear evidence of the genotoxic potential of cypermethrin. Aberrations of mitotic cycle, change of mitotic index and chromosomal abnormalities observed after exposure to toxic metals, metalloids or organic pollutants were attributed to the disorganization and depolymerization of microtubules, which underlie these processes in higher plant cells (Liu et al., 2009). The wide range of observed abnormalities is more evident in metaphase and anaphase. This suggests that even short term exposure to relatively small concentrations of cypermethrin can significantly (p≤0.05) affect the mitotic index, chromosome structure and disturbs mitotic spindle formation. The chromosomal fragments according to Hemavathi et al. (2015) can be attributed either to chromosomal breakages in anaphase bridges and metaphase due to cohesive chromosomal translocations or multiple breakages of the chromosome due to loss of chromosome integrity. The chromosome bridges seen is an indication of clastogenic effect caused by chromosome breaks, vagrant chromosomes and c-metaphases show an increased risk for aneuploidy in the plant while sticky chromosomes is an indication of a highly toxic, irreversible effect which may lead to cell death (Ping et al., 2012).

Mitotic index of root tips treated with cypermethrin decreased significantly as the exposure duration increased. This shows that the inhibition of mitosis in Allium sativum roots by cypermethrin is dependent on the treatment time. However, reduction in mitotic index was greater across concentration than across exposure duration. The lowering of mitotic index in the treated root tips could be due to inhibition of DNA synthesis (Sudhakar et al., 2001), arrest of one or more mitotic phases (Kabarity et al., 1980) or blocking of G2 phase in the cell cycle (El-Ghamery et al., 2000) preventing the cell from entering mitosis. The reduction in mitotic activity with increasing concentrations clearly demonstrated the ability of the pesticide to inhibit DNA synthesis.

The results obtained from the study indicate the genotoxicity of cypermethrin to A. sativum root meristem cells with even very minute concentration of the chemical showing significant toxicity to the cells. Also, chromosomal aberrations induced by cypermethrin are not dose-dependent as aberrations depend on the number of cells undergoing mitosis. Having obtained a cypermethrin concentration that produced result with lower cytotoxic effects than the concentration currently used on farms, it is therefore recommended that entomologists should test the efficacy of this concentration on pests. This concentration is below the EC_{50} value and may pose less threat to man, animals and plants.

In conclusion, this study has validated the utility of root cells of A. sativum for monitoring the genotoxic effects of cypermethrin and enabled the assessment of genetic endpoints such as mitotic index and chromosome aberration.

REFERENCES


Dallhoff, K. 2018. Aquatic toxicity of Cypermethrin. Thesis PhD. Department of Plant and Environmental Sciences, Faculty
of Science, University of Copenhagen. https://www.forskningsdatabasen.dk/en/catalog/243882


Identification of anti-inflammatory compound/compounds in hexane fraction of Jatropha curcas root extract

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Abstract. Jatropha curcas is a medicinal plant with many therapeutic properties such as anti-inflammatory, anti-malaria, anti-cancer and antioxidant. The root extract has been shown to possess high anti-inflammatory activity. Previously, the compounds responsible for this activity have not been fully elucidated. Two fractions (Fraction 1 and Fraction 2) obtained from a preparative HPLC of the root extract showed significant anti-inflammatory and cytotoxic activities in RAW 264.7 murine macrophage cells with Fraction 1 giving higher nitric oxide (NO) inhibition compared to Fraction 2 and L-NAME. Further purification steps involving column chromatography, thin layer chromatography and analytical HPLC of Fraction 1 produced two fractions labeled as Fraction A and Fraction B. Both fractions showed anti-inflammatory activity without cytotoxic activity in RAW 264.7 cells. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis showed that Fraction A contained a group of 18 carbon fatty acid compounds consisting of 2 oxooctadecanoic acids; 15, 16 dihydroxy 9Z, 12Z octadecadienoic acid; octadecadienoic isomer and 15,16 dihydroxy 9Z, 12Z octadecadienoic acid, 15S, 16S. The 18-carbon fatty acid structure was confirmed by nuclear magnetic resonance (NMR) spectral data. The IC_{50} value of compounds in Fraction A for anti-inflammatory activity in RAW 264.7 cell line was 434.8±0.75 µg/mL. From the analysis, it can be concluded that Fraction A can be classified under 18 carbon long chain fatty acid group based on LC MS/MS and NMR analysis. This active compound shows an inhibition towards NO activity.

Keywords: Jatropha curcas, anti-inflammatory, C 18 fatty acids, octodecanoic acid, IC_{50}

INTRODUCTION

Jatropha curcas Linn. belongs to the Euphorbiaceae family with about 170 known species (Sabandar et al., 2013). The word Jatropha was derived from two Greek words of “jatris” means “doctor” and “trophé” means “food” (Kumar and Sharma, 2008). Although it was a native plant of Mexico
and Central America, the plant is now widespread in other parts of the world including South-east Asia, India and Africa. It has gained importance in Malaysia as a source of seed oil for biofuel production. In many African and Asian countries, J. curcas plant is considered a traditional herb to cure various ailments ranging from simple fever to infectious diseases, including sexually transmitted (Pandey et al., 2012). In the tropics and Central America, Jatropha seeds are used as a purgative and supplement in tea drinks. However, this practice was believed to cause strong irritation, poisoning and cancer (Adolf et al., 1984). A research conducted in Sri Lanka showed that Jatropha seeds could also be used as a pregnancy abortifacient (Goonasekera et al., 1995). However, in Mexico, the seeds are roasted or boiled before being used as an ingredient in traditional cuisines (Makkar et al., 1998).

The Jatropha leaves have been used to treat various illnesses. The Mayan used leaves parts to treat rashes and bone fracture (Gomerford, 1996). In Ghana, the leaves were used for anti-malarial treatment by boiling the leaves and using the extract as a drink (Asase et al., 2005). The plant leaves had also been reported to be low in sodium and considered appropriate for a person who practiced low sodium diet (Méndez et al., 2014). The latex from Jatropha tree also possesses medicinal property, whereby in Indonesia, the latex was used to treat infected wounds and ulcer (van den Berg et al., 1995). The Jatropha root is also useful in the treatment of certain diseases. A report from Brazil claimed that the extract from Jatropha rhizome could treat Schistosomiasis mansoni disease that is caused by S. mansoni worm present in a fresh-water mollusk Biomphalaria glabrata which infects the liver (dos Santos and Sant’Ana, 1999). Another property of Jatropha root extract is the anti-inflammatory activity on RAW 264.7 macrophage cells (Mujumdar and Misar, 2004; Nayak and Patel, 2010). Our previous report (Othman et al., 2015) showed that the anti-inflammatory activity of J. curcas root was due to the presence of fatty acids. However, the nature of the active fatty acids was unknown. Thus, the present study was conducted to elucidate the structure of the active fatty acids involved in the anti-inflammatory activity.

MATERIALS AND METHODS

Purification of compounds by chromatographic methods

Jatropha curcas L. roots were obtained from Farm 2, Faculty of Agriculture, Universiti Putra Malaysia (UPM). The plant was verified by the botanist, Dr. Shamsul Khamis at the Institute of Bioscience, UPM and deposited in the Phytomedical Herbarium, Institute of Bioscience, UPM with voucher number SK1764/2010. The root sample was extracted with 80% methanol, followed by liquid partition chromatography (Othman et al., 2015). The hexane fraction which showed high anti-inflammatory activity was subjected to preparative high-performance liquid chromatography (HPLC). Two fractions (Fraction 1 and Fraction 2) obtained showed significant anti-inflammatory and cytotoxic activities in RAW 264.7 murine macrophage cells. The fractions were further purified by a mini open column (1x4 cm), packed with silica gel 60:70-230 Mesh (ASTM) and prewashed with hexane: ethyl acetate (EtOAc) (7:3). A sample of 1 mL (20.0 mg/mL) was loaded and eluted with 3 mL of hexane:EtOAc (7:3). The mobile phase, consisted of 20 mL hexane:EtOAc solution (6:4) was used to collect 1 mL eluates. The eluates were subjected to analytical thin layer chromatography (TLC) on silica gel 60 F<sub>254</sub> (0.25 mm thickness) coated plates and developed in hexane:EtOAc (6:4). The spots were viewed under UV at the wavelength of 254 nm (short wave) and 360 nm (long wave). The spots with similar profiles were pooled and air dried. The dried samples were first dissolved in hexane and were analyzed by analytical HPLC. A JASCO Liquid Chromatography System consisting of a JASCO Pump PU-2089 Plus and JASCO Absorbance Detector model UV-2077 Plus 4-Intelligent UV/VIS Detector linked by JASCO BORWIN version 1.5 software was used. The analysis was conducted by using an Agilent column, ZORBAX Eclipse XDB-C18 (4.6 x 250 mm: 5 μm). The solvent system was MeOH:H<sub>2</sub>O in a ratio that ranged from 2% to 100% of H<sub>2</sub>O in a gradient mode at a flow rate of 1.0 ml/min. The gradient was set at 2% of H<sub>2</sub>O from 0 to 5 minutes, continuously increasing the water percentage to 5% from 5 min to 20 min. After 20...
minutes, the water percentage was increased to 20% for 10 min before 100% MeOH was used to flush the column for 30 min. Two fractions (Fraction A and Fraction B) obtained from this analysis were subjected to anti-inflammatory and cytotoxic assays.

**Compound analyses**
Fraction A which showed higher anti-inflammatory activity without cytotoxic activity than Fraction B was used for identification of compounds present in the fraction. The fraction was analyzed by Liquid Chromatography Tandem Mass Spectrometry (LCMS/MS) and Nuclear Magnetic Resonance (NMR). LCMS/MS analysis was done using AB Scix 3200QTrap LCMS with Perkin Elmer FX 15 UHPLC system. An Agilent Zorbax C18 (150 mm x 4.6 mm: 5um) was used as the column. The mobile phase consisted of A (water) and B (acetonitrile). Both solvents A and B contained 0.1% formic acid and 5 mM ammonium formate. A gradient was programmed to 10% B to 90% B from 0.01 to 8.0 min, hold for 2 min and back to 10% B in 0.1 min with re-equilibration for 5 min. The sample volume was set at 20 µL per injection.

Proton-1 and carbon-13 NMR analyses were carried out on Varian NMR system 500 MHz at 500 and 125 MHz respectively. The signals obtained were described in terms of chemical shifts (δ ppm), multiplicity, coupling constants and a number of protons and carbons where appropriate. Both 1D and 2D NMR were measured in deuterated chloroform (CDCl₃).

**Anti-inflammatory activity, cytotoxic assay and IC₅₀ value determination**
Murine monocytic macrophage RAW 264.7 cell were cultured in Dulbecco's Modified Eagle Media (DMEM) with 4 mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate and 10% of fetal bovine serum (FBS). The cells were incubated in a 50 ml culture flask in a CO₂ incubator with 5% CO₂ at 37°C. Then the RAW 264.7 cells were seeded in a 96-well microplate (1x10⁴ cell/mL) and incubated in 5% CO₂ at 37°C for 2 to 3 hours. Different volumes with final concentration (1.0, 0.5, 0.25, 0.125 mg/mL) of sample were diluted in DMSO were added to the wells by serial dilution for IC₅₀ determination. The cells were then stimulated by adding 100 U/mL of IFN-γ and 5 ug/mL of LPS from *Escherichia coli* strain 055:B5. The cells were incubated in 5% CO₂ at 37°C for 18 hours. The nitric oxide (NO) concentration produced by RAW 264.7 cell was determined by Griess assay (Syahida et al., 2006). N-nitro-L-arginine-methyl ester (L-NAME) which acted as iNOS inhibitor was used as a positive control at a concentration of 250 µM. The cytotoxic effect of the sample was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Syahida et al., 2006). All experiments were done in triplicate.

**Statistical analysis**
All data were subjected to one-way analysis of variance (ANOVA). Treatment means were compared using Tukey's multiple comparison tests. The IC₅₀ value was determined by using one-phase decay analysis. Statistic software Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA) was used for all statistical analyses.

**RESULTS AND DISCUSSION**

**Anti-inflammatory activity of Fraction A and Fraction B**
The analytical HPLC analysis produced two fractions labeled as Fraction A and Fraction B. Fraction A showed a higher nitric oxide (NO) inhibition than Fraction B and L-NAME as shown in Figure 1a. Both fractions were non-toxic towards RAW 264.7 cells growth (Figure 1b). The purification steps conducted with the methanolic extract of *J. curcas* produced an active anti-inflammatory fraction without cytotoxicity activity. The non-toxic property is an important consideration as the anti-inflammatory assay was based on the measurement of NO production and a decreased in NO quantity could also mean cell death. As shown by the MTT assay from previous analysis, the hexane fraction caused cell death, while the Griess assay showed a reduction in NO production (as an indication of anti-inflammatory activity) of the RAW 264.7 macrophage cells (Othman et al., 2015). After a series of purification steps, the fractions obtained (Fraction A and B), showed an anti-inflammatory activity and were non-toxic towards RAW 264.7 macrophage cells. This indicates that the compounds extracted were
actually inhibiting the NO production of the RAW 264.7 macrophage cells through inhibition of NO signaling pathway.

**Figure 1a.** Anti-inflammatory activity of Fraction A and Fraction B. Fraction A exhibited a higher anti-inflammatory activity compared to Fraction B and L-NAME. L-NAME and induced cells represented the positive and negative control, respectively. Each histogram represents a mean of three replicates with error bar representing the standard deviation. *Indicates significant difference (P<0.05) compared to the experiment control.

**Figure 1b.** Cytotoxic activity of Fraction A and Fraction B. Both fractions showed no cytotoxic activity towards RAW 264.7 murine macrophage cells growth. Each histogram represents a mean of three replicates with error bar representing the standard deviation. *Indicates significant difference (P<0.05) compared to the experiment control.

**Elucidation of active compound by LCMS/MS and NMR**

Analysis by LCMS/MS negative mode showed four major peaks in Fraction A that belonged to long chain fatty acid group (Figure 2). The data were then compared with the databases of ACD/Labs advanced chemometrics mass fragmentations predictive software. According to the database analysis, the four compounds with almost similar m/z values belonged to the octadecadienoic acid and an octadecanoic acid group (Figure 2). The compounds nomenclature and their mass values are shown in Table 1. The compounds identified were 12 oxooctadecanoic acid (Figure 2a), a substituted saturated C18 fatty acid; 15, 16 dihydroxy 9Z, 12Z octadecadienoic acid (Figure 2b), a substituted unsaturated C18 fatty acid; octadecadienoic isomer (Figure 2c), an unsaturated C18 fatty acid and octadecanoic acid isomer (Figure 2d), a saturated C18 fatty acid.

The chemical structures of compounds in Fraction A were confirmed by NMR analysis. The NMR data which were analyzed by using ACD/NMR software from ACD/Labs.com are presented in Table 2. The peak signals were assigned at δH 0.88 (t, J=6.6 Hz, 2H), 1.26 (br.s., 28H), 1.63 (m, 2H), and 2.34 (t, J=7.5 Hz, 2H). Proton-1 and carbon-13 peak analysis are presented in Figure 3a and 3b, respectively. Although, a fifth peak was observed in the LCMS/MS spectrum, but the structure was not verified by NMR spectral data. Hence, the compound was unidentified.

The LCMS/MS analysis showed the presence of four compounds as members of the C18 fatty acids. The correlations of C-H according to the NMR data were in agreement with the structure belonging to a long chain fatty acid due to the presence of carboxylic group peak at 180 ppm. No aromatic group was observed in the C13 analysis. Hence, the results of both LCMS/MS and NMR analyses were in agreement that the compounds present in the active Fraction A were C18 fatty acids.

However, the results obtained in the present study could not identify the specific compound responsible for the anti-inflammatory without cytotoxic activity. It is possible that all the fatty acids identified possessed anti-inflammatory activity as previous reports have shown that long chain fatty acids such as linolenic acid, linoleic

Anti-inflammatory compounds in Jatropha curcas root

Acid, palmitoleic acid and octadecadienoic acid possessed anti-inflammatory activity (Ballou and Cheung, 1985; Mathavi and Velavan, 2013), these fatty acids were proven to regulate the anti-inflammatory pathway (Aparna et al., 2012). The anti-inflammatory mechanism of fatty acid was shown by using enzyme kinetic study and X-ray crystallography analysis (Kudo and Murakami, 2002). It was reported that the carboxylic group from saturated fatty acid binds to the amino acid Asn 67 of the hydrophobic region of the phospholipase A2 (PLA2) active site (Kudo and Murakami, 2002). Based on the fatty acid mechanism explained earlier, it can be assumed that fatty acids competed with PLA2 substrate, thus acting as a competitive inhibitor towards PLA2. This enzyme is involved in catalyzing the release of arachidonic acid, a precursor for the synthesis of inflammatory mediators at the sn-2 position of membrane phospholipid (Diaz and Arm, 2003; Calder, 2004).

Table 1. Compounds identified by LC-MS/MS in Fraction A.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 Oxooctadecanoic acid (Figure 2a)</td>
<td>183.1</td>
</tr>
<tr>
<td>15,16-Dihydroxy-9Z,12Z-octadecadienoic acid (Figure 2b)</td>
<td>183.0</td>
</tr>
<tr>
<td>Octadecadienoic acid isomer (Figure 2c)</td>
<td>184.0</td>
</tr>
<tr>
<td>Octadecanoic acid isomer (Figure 2d)</td>
<td>183.0</td>
</tr>
</tbody>
</table>

Table 2. 2D-NMR spectral data of Fraction A.

<table>
<thead>
<tr>
<th>δ_H (in ppm)</th>
<th>δ_C (in ppm)</th>
<th>COSY (in ppm)</th>
<th>HMBC (in ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.88, 2H, t, J = 6.6</td>
<td>14.1</td>
<td>1.63 (s), 1.26</td>
<td>22.6, 31.9</td>
</tr>
<tr>
<td>1.26, 28H, br.s (Overlapped)</td>
<td>22.7, 24.7, 29.7, 31.9</td>
<td>1.63</td>
<td>14.1, 22.6, 31.9</td>
</tr>
<tr>
<td>1.63, 2H, m, J = 7.5</td>
<td>24.7</td>
<td>2.34</td>
<td>180, 34.1</td>
</tr>
<tr>
<td>2.34, 2H, t, J=7.5</td>
<td>34.1</td>
<td>1.26, 1.63</td>
<td>180, 29.7, 24.7</td>
</tr>
</tbody>
</table>

Figure 2. LC-MS/MS spectrum. Compounds 1 to 4 of Fraction A were assigned based on comparison to databases.
Inhibitory concentration of active compounds in Fraction A

Inhibitory concentration (IC$_{50}$) indicates the amount of sample concentration that could inhibit 50% of NO production in RAW 264.7 macrophage cells. A series of sample concentrations ranging from 0.0 to 1.0 mg/mL were evaluated. Sample absorbance was measured at 550 nm and IC$_{50}$ graph was plotted using Graphpad Prism 5 software. The IC$_{50}$ value of the active compounds in Fraction A was determined to be 434.8±0.75 µg/mL (Figure 4).

Inhibitory concentration assay of Fraction A showed an IC$_{50}$ value of 434.8±0.75 µg/mL in murine macrophage RAW 264.7 cells. In an earlier study, Oskouieian et al. (2011) reported a concentration of 3.1 µg/mL of methanolic extract of $J$. curcas root could inhibit NO production almost completely in RAW 264.7 macrophage cells (Oskouieian et al., 2011). However, the latter authors also observed that the crude extract was toxic to the cells. This raised the question whether NO inhibition was actually the result of cell death or the anti-inflammatory activity per se. On the other hand, it was clearly shown in the present study that the isolated compounds in Fraction A from the $J$. curcas root were non-toxic to the RAW 264.7 macrophage cells. Even though the IC$_{50}$ value for the present study was higher compared to the previous report, it proved that the inhibition of compounds in Fraction A was due to the inhibition of NO signaling pathway and not by the toxicity effect towards RAW 264.7 growth.

The presence of active compounds which showed NO inhibition from other plant sources has been reported. A crude extract from $P$. dentata (seaweed) showed more than 95% inhibition of NO production at 200 µg/mL with more than 90% of cell viability (Kazłowska et al., 2010), while extract of $P$. macrocarpa, (a tropical herbal plant), showed the highest inhibition of NO production in RAW 264.7 cells at 3.13 µg/mL (Hendra et al., 2011). A hexane fraction prepared from $O$. japonicas at 100 µg/mL showed inhibition of NO production in RAW 264.7 cells with cell viability over 80% (Lee et al., 2013). These inhibitory concentrations were lower than the value observed in the present study. This is not surprising as crude extract might contain a complex mixture of compounds that would possibly work synergistically to give a potent anti-inflammatory activity compared to a single compound.
CONCLUSION

The nature of compounds presents in J. curcas root methanolic extract which possessed anti-inflammatory without cytotoxicity activity in RAW 264.7 macrophage cells in vitro was elucidated. The compounds were identified as long chain fatty acids with 18 carbons based on mass spectrophotometry and NMR analysis. The IC_{50} value of Fraction A in NO inhibition was 434.8±0.75 µg/mL.

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REFERENCES


Molecular identification and characterization of medically and veterinary important flies of Bangladesh based on mitochondrial COI gene sequences

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Abstract. Flies are considered serious pests which cause health problems of human and animal, transmitting many pathogenic microbes. Pest management programs depend on proper identification of pests. The present research work is an initiative to identify the medically and veterinary important flies based on mitochondrial COI gene sequences. Eleven species of the fly pests were identified. Among them, four fly species were the first record from Bangladesh. The phylogenetic analysis of retrieved sequences confirmed that the evolution of these species occurred from a common ancestor. Highest AT percentage (69.9%) was found in Haematobia irritans exigua and lowest GC percentage (30.4%) was found in Haematobia irritans exigua. The substitution rate of codon was found 1.88 in 1st position, 0.73 in 2nd position and 1.22 in 3rd position, respectively. Interspecific genetic divergence range of flies sequences was 5-20%. Haplotype network showed that Atylotus agrestis was mostly diverged from its common ancestors by 37 mutational steps. This research is the first molecular approach to identify the medically and veterinary important flies based on MT-COI gene sequences along with the establishment of first DNA barcode dataset for accurate identification in Bangladesh.

Keywords: medically and veterinary important flies, MT-COI gene, phylogenetic analysis, interspecific divergence, haplotype

INTRODUCTION

Dipteran flies exist as a major pest of humans, poultry and livestock facilities throughout the world (Greenburg, 1973; Geden et al., 2006; Iqbal et al., 2014; Rahaul, 2013). Flies are the veterinary, medically and economically important because both sexes feed on the blood of large mammals, often humans (Sukontason et al., 2008). The flies pick up disease-causing organisms while crawling and feeding. Transmission takes place when the fly contacts people or their food (Bhakdeenuan et al., 2012). Their painful biting can also cause serious irritation, pain and stress to livestock resulting in significant decreasing in weight loss and milk yield (Sheppard et al., 1990).

Synanthropic flies can cause annoyance and act as carriers of mechanical transmitting pathogens to human (Lane and Crosskey, 1993). They are responsible for the spread of acute gastroenteritis, trachoma among infants and young children in developed countries and spread of nosocomial infections with multiple antibiotic-resistant bacteria in the hospital environment.
(Graczyk et al., 2001). Flies act as a mechanical vector of some enteric bacteria named Saurocystis
sp., Toxoplasma gondii, Isospora sp., Escherichia coli, Schigelia, Salmonella sp., Mycobacterium sp., Bacillus
anthracis, Enterobacter sakazakii can transmit more than hundred diseases in humans and animals
(Adenusi and Adewoga, 2013; Fetene et al., 2011; Mian et al., 2002; Mramba et al., 2007; Macovei et
al., 2008; Scott et al., 2014; Tian et al., 2011). Adult flies can carry intestinal worm eggs viz. Ascaris sp.,
hookworm, Trichuris trichura, Oxymiuris vermicularis, Taenia solium, Taenia saginata, Mycobacterium
tuberculosis which causes pathogenicity in human (Preativatanyou et al., 2010; Sembel, 2010).

Identification of flies is essential for both epidemiological study and control strategies
(Bhakdeeuan et al., 2012; Greenberg and Kunich, 2002; Siritawanarungsee et al., 2005; Sukontason
et al., 2006; 2008a; 2008b; Tumrasvin et al., 1979). The problem of morphological identification is
that there are no taxonomic key coverings all stages of fly species at present (Wells and Sperling,
2001). The morphological similarity in females of a certain species complex of insects including flies
is a challenge for identification (Archana et al., 2016). In order to solve the problems of taxonomic identification, molecular techniques such as nucleotide sequence analysis were proposed (Stevens and Wall, 2001).

A novel methodology known as DNA barcoding has the potential to mitigate challenges
posed by identification of insect pests (Quicke et al., 2012; Sethusa et al., 2014). DNA barcoding is
gaining broad application in Integrated pest management (IPM) program as the standard method for species identification by matching unknown gene against the known (Etzler et al., 2014). A short standard region of the genome, specifically the mitochondrial gene, mitochondrial cytochrome c oxidase subunit I gene (MT-CO1), is used in most of the cases (Hebert et al., 2003; Sharma et al., 2015) There is scarcity of information on medically and veterinary important flies especially through DNA barcoding in Bangladesh.

This study was therefore aimed to investigate molecular characterization and identification of medically and veterinary important flies of Bangladesh through establishing DNA barcode dataset.

MATERIALS AND METHOD

Fly sampling
Medically and veterinary important flies were collected with the help of sweeping net mainly from food courts, dumping ground, cowsheds, butchery and poultry farm of various regions of Bangladesh. The collected flies were placed in -20°C freezer to kill them. Species of all specimens were identified by using morphologically taxonomic keys (Carvalho and Mello-Patui, 2008; Emden, 1939; Whitworth, 2010; Zimin and Elberg, 1988) under a stereomicroscope. Somatic tissue rich in mitochondria (e.g. legs and wings) were separated carefully from the specimens, cleaned by ethanol and kept for molecular analysis. The rest of the body of each fly was stored in -20°C freezer as a voucher specimen.

DNA extraction
The genomic DNA was extracted from leg and wings tissues using Wizard® Genomic DNA
Purification Kit, (Promega, USA) following the manufacturer’s protocol. The concentration and purity of DNA were measured by using Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20°C until further use.

PCR amplification, gel electrophoresis and sequencing
The genomic DNA extract was subjected to PCR amplification of a 710 bp region near the 5’
terminus of the COI gene in a thermal cycler 96 well plates (Veriti, Applied biosystems by Thermo
fisher Scientific, USA) with 20 μl reaction volume contains 10 μl Master mix (GoTaq® Green
Master Mix, Promega), 1 μL (10 pmol) forward primer, 1 μL (10 pmol) reverse primer, template
DNA 50 ng and adjustable nuclease free water. Forward primer LCO1490(F)-5’GGTCAACAAA
TCATAAAGATATTGG-3′ and reverse Primer
HCO2198(R)-5’-TAAACTTCAAGGGTGAACCA
AAAATCA-3′ (Folmer et al., 1994) which amplify
a 710 bp segment were used. COI gene was amplified using PCR protocol, as follows: Initial
step: 94°C for 3 minutes, 32 cycles of the following profile: Denaturing step: 94°C for 30
seconds, Annealing step: 49°C for 30 seconds, Extending step: 72°C for 45 seconds. The
amplified product was analyzed on a 1% agarose gel electrophoresis. The PCR product was purified using Promega Wizard® SV Gel and PCR clean up system (Promega Corporation, USA). The sequencing reaction was sent to Apical Scientific Sdn. Bhd, Malaysia and performed using ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA).

**Sequence analysis**

After proper editing of sequences using FinchTV software, all the sequences were deposited in the NCBI GenBank (BankIt) to obtain the accession numbers for all these sequences (Table 1). Some sequences were downloaded from NCBI GenBank for Phylogenetic analysis.

**Table 1. GenBank accession numbers and the GPS position of the sequenced fly species.**

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<thead>
<tr>
<th>Species Name</th>
<th>Latitude, Longitude</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Musca domestica</em></td>
<td>23.87565N, 90.267075E</td>
<td>MG587909</td>
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<tr>
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<td>23.52228N, 90.16414E</td>
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<td><em>Musca inferior</em></td>
<td>23.77138N, 90.373340E</td>
<td>MH086026</td>
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<tr>
<td><em>Musca sorbens</em></td>
<td>23.765874N, 90.377806E</td>
<td>MH666047</td>
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<td>21.838507N, 90.24927E</td>
<td>MH427032</td>
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<td>23.876879N, 90.266066E</td>
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</table>

**Bioinformatic analysis**

COI sequences were aligned using ClustalW algorithm with the help of MEGA tools (version 6) with gap opening penalty 15, gap extensions penalty 6.66, transition weight 0.5 and delay divergent cutoff 30% (Kobayashi et al., 1998; Simon and Hadrys, 2013). Multiple sequence alignment images were prepared using Jalview, version 2.9 (Von der Schulenburg et al., 2011). For calculation of nucleotide base components, MEGA 6 software was used. For calculation of genetic distances among sequences, Kimura’s two-parameter method (K2P) of base substitution was used in MEGA 6 (Tamura et al., 2013). The rate of transitions (TS) and transversions (TV) at the first, second and third codon positions were calculated and plotted against the F48 genetic using DAMBE 5.3.10 (Xia, 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Haplotypes were constructed using the program Pop art 1.7 based on TCS network (Clement et al., 2000).

RESULTS

A total of 1156 fly specimens were collected from various regions of Bangladesh. The MT-COI gene of flies were sequenced and BLAST (Basic Local Alignment Search Tool) was used to check sequence identity between the retrieved sequences and GenBank library or database of sequences from NCBI GenBank. This helps to identify sequence similarity across genomes. They were found belong to 11 species named *Musca domestica, Synthesiomyia nudiseta, Musca inferior, Musca sorbens, Haematobia irritans exigua, Atylotus agrestis, Chrysomya megacephala, Chrysomya rufifacies, Hemipyrellia ligurriens, Sarcophaga ruficornis* and *Sarcophaga misera*. Among them, *Synthesiomyia nudiseta, Chrysomya rufifacies, Musca inferior* and *Hemipyrellia ligurriens* were reported for the first time from Bangladesh.

**Nucleotide composition**

Nucleotide composition analysis was performed by MEGA, 6 version to know the percentage of base contents (Table 2). Highest AT percentage (69.9%) was found in *H. irritans exigua* and highest GC percentage (34.3%) was found in *A. agrestis*. Lowest GC percentage (30.4%) was found in *H.
irritans exigua and lowest AT was found in (65.7%) in A. agrestis.

**Rates of substitutions**
The three codon positions of the COI gene sequences of flies were tested for saturation by plotting the number of transitional and transversional substitutions against the F48 for all the three codons and were represented as a 3D plot. The plot showed the transition and transversion for the first and third codon position increased along the F48 distance. But, the substitutions in the second codon position was less. Exact saturation of the substitution rate did not occur in all the three codon positions. In 1st codon position, estimated transition/transversion ratio was 1.88, in 2nd codon position, estimated transition/transversion ratio was 0.73 and in 3rd codon position, estimated transition/transversion ratio was 1.22 (Figure 1).

**Genetic distance**
The interspecific genetic distance range of flies varies between 5-20%. Highest interspecific distance 20% was observed between S. misera and A. agrestis. Lowest 5% genetic distance was found between M. domestica and M. sorbens (Table 3).

**Table 2.** The nucleotide base composition of the sequenced fly species.

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<th>C</th>
<th>A</th>
<th>G</th>
<th>AT(%)</th>
<th>GC(%)</th>
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Figure 1. Medically and veterinary important flies’ number of substitutions (S= transitions, V= transversions) in Y axis against F48 genetic distance, in X axis for each codon position represented as A, B and C.
Table 3. Interspecific genetic distance of medically and veterinary important flies.

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</table>

Multiple sequence alignment
Multiple sequence alignment was performed by Jalview 2.9. Conserve region of sequences were presented by dots and non-conserved region was presented by letter (Figure 2).

Haplotype analysis
Haplotype analysis of mitochondrial COI gene of flies showed high genetic diversity among them. Sarcophaga ruficornis and S. misera was separated from its immediate ancestor by 19 and 26 mutational steps, respectively. Chrysomya rufifacies was separated from its immediate ancestor by 21 mutational steps. Chrysomya megacephala and Syn. nudiseta were separated from its immediate ancestors by 17 and 37 mutational steps respectively. Haematobia irritans exigua and A. agrestis were separated from its common ancestor by 19 and 53 mutational steps respectively. Musca sorbens and M. inferior were closely separated from its common ancestor by 15 and 28 mutational steps respectively. Musca domestica diverged from its immediate ancestor by 29 mutational steps (Figure 3).

Phylogenetic analysis
Phylogenetic analysis revealed that species belong to same family were in same clade (Figure 4). Sarcophaga misera and S. ruficornis belong to Sarcoptidae family where they were closely related and shared 99% sequence identity. Chrysomya megacephala and C. rufifacies shared 51% sequence identities between them. Musca inferior separated from Musca domestica and M. sorbens. Musca domestica and M. sorbens shared 45% sequence similarity between them. Synthesiomyia nudiseta were separated from others and shared 43% sequence identity. Atylotus agrestis were in the different clade than other fly species.
Figure 2. Multiple sequence alignment of flies. Dots denote the conserved region and letter denotes the non-conserved portion among these eleven nucleotide sequences of flies.

Figure 3. Mitochondrial COI gene haplotype analysis of fly species constructed by Popart 1.7 based on TCS network. Big circles represent the haplotype and small circles represent the immediate common ancestors. Mutational steps are presented by hatch marks and numbers.
Figure 4. Evolutionary relationship among flies was built by MEGA6. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood was -2091.0319. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together was shown above the branches. The bottom bar 0.05 denoted the genetic change. *Apis mellifera* was considered as outgroup.
DISCUSSION

During the present study, a total of 1156 fly specimens were collected from different parts of Bangladesh. We have sequenced and identified eleven (11) medically and veterinary important fly species. The medically important fly species were *Musca domestica*, *M. inferior*, *M. sorbens*, *Syn. nudiseta*, *C. megacephala*, *C. rufacies*, *H. ligurriens*, *S. ruficornis* and *S. misera*, these species can transmit typhoid, cholera, tuberculosis, skin myiasis etc. in human. *Haematobia irritans exigua*, *Atylotus agrestis* were the veterinary important flies. *Haematobia irritans exigua* biting was responsible for loss of blood, and results in detrimental effects on host physiology as to include reduction in milk production, efficiency, and rate of gain (Onder et al., 2018). *Atylotus agrestis* transmits the Trypanosoma conglense in cattle (Desquesnes and Dia, 2003).

Among sequenced flies, *Syn. nudiseta*, *C. rufacies*, *M. inferior* and *H. ligurriens* were the first report from Bangladesh. Ferdousi et al. (2004) recorded six medically and veterinary important flies named *Musca conduscens*, *Musca ventrosa*, *Musca sorbens*, *Stomoxys calcitrans*, *Hematobia irritans exigua* and *Tabanus striatus* in savar region of Bangladesh based on morphological keys. In Iran, 10 species of medically important flies were identified based on morphological keys (*Lucilia sericata*, *Chrysomya megacephala*, *Chrysomya albiceps*, *Calliphora vicina*, *Sarcophaga aegyptica*, *Sarcophaga africa*, *Wohlfartia magnifica*, *Passeromyia* species, *Muscina stabulans* and *Musca domestica*) (Khoodbel et al., 2013). Identification of pest based on morphological characteristics may cause erroneous identification. However, limited research was found on molecular identification of flies. Archana et al. (2014) reported molecular identification of six fly species named as *Chrysomya imicola*, *C. oxyzoma*, *C. peregrinus*, *C. anopheles*, *C. palpiifer*, and *C. bifini*. Singh and Achint (2017) reported *M. sorbens*, *M. domestica*, *H. irritans*, *M. crassirostris*, *S. calcitrans* as medically important flies from four districts of Punjab of India.

Sequence alignment is an important method in bioinformatics for visualizing the relationships among organisms (Kashmeera and Shudhikumar, 2015). We observed A-T base content was higher than G-C base content in mtDNA of fly species. This was caused by A-T bond, which has a noncoding region that has further evolution rate compared to the coding region. Highest AT percentage (69.9%) was found in *H. irritans exigua* and highest GC percentage (34.3%) was found in *A. agrestis*. Lowest GC percentage (30.4%) was found in *H. irritans exigua* and lowest AT (65.7%) was found in *A. agrestis* (Table 2). Bajpai and Tewari (2010) found that AT content was higher in fly species which was similar to our findings. The average nucleotide composition for the fly species was T=40.58.6%, A=30.10%, C=16.08%, G=13.24%.

We observed substitutions against the F48 for all the three codons of flies (Figure 1). In 1st codon position, estimated transition/transversion ratio was 1.88. In 2nd codon position, estimated transition/transversion ratio was 0.73 and in 3rd codon position, estimated transition/transversion ratio was 1.22. The findings of Karthika et al. (2016) also supports our results.

We found interspecific genetic distance range of flies was 5-20% (Table 3). Sharma et al. (2015) studied ten Indian flesh fly species and showed the interspecific genetic divergence of 4-14%. Guo et al. (2011) reported interspecific variation varying from 7% to 10% in Sarcophagid species.

Haplotype analysis of mitochondrial COI gene of medically and veterinary important flies showed high genetic diversity among them (Figure 3). *Atylotus agrestis* was mostly diverged among all other fly species because of its highest mutational steps.

Phylogenetic analysis revealed that species belong to same family were in the same clade (Figure 4). Singh and Achint (2017) found that *Musca* species formed a sister clade with *Haematobia irritans* which supports our results. Iwasa and Ishiguro (2010) reported *Haematobia* and *Musca* as sister clade similar to our findings.

CONCLUSION

This is the first study of medically and veterinary important fly species of Bangladesh with some new reports based on mitochondrial COI gene. Identification of different fly species was confirmed based on COI gene to prove their correct identity. The phylogenetic analysis has led to confirm that evolution of these species has
occurred from a common ancestor and the species are closely related to each other. The current study provides a useful reference backbone for research on medically and veterinary important flies based on MT-COI gene sequences through establishing DNA barcode dataset.

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REFERENCES


Synthesis and characterisation of chitosan nanoparticle as a potential delivery carrier

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Abstract. Chitosan is a biodegradable, non-toxic polysaccharide that is extensively studied as a biocompatible vector for gene and drug delivery. However, the fabrication of chitosan nanoparticle (CNP) is usually encountered with a wide size distribution and poor particle stability, which unfortunately limits their role for certain biological applications. This study reports the synthesis and characterisation of CNPs under optimised conditions. The CNPs were synthesised via ionic gelation process utilizing tripolyphosphate (TPP) as a cross-linking agent. The particle size and morphology of samples were subsequently evaluated using dynamic light scattering (DLS), electron microscopy and Fourier-transform infrared spectroscopy (FTIR). Findings arising from this study showed the optimised nanoparticles exhibited spherical shaped CNPs with a size range from 4 to 25nm which lays the foundation for further applications.

Keywords: Chitosan nanoparticles, dynamic light scattering, Fourier-transform infrared spectroscopy, transmission electron microscope

INTRODUCTION

The delivery of genes and nucleic acid, especially non-viral routes have been developed as a powerful and popular approach for gene therapy and DNA vaccination in the treatment as well as control of diseases that are likely to impact clinical medicine and biotechnology (Luo and Saltzman, 2000). The ability to transfer foreign DNA into cells safely and efficiently is a fundamental goal for such applications. Therefore, there lies a growing need to improve its current effectiveness in terms of efficient delivery and sustained antigen release. In order to achieve this, research has greatly skewed towards the use of biodegradable polymers as nanoparticle-based carriers to deliver and increase intracellular accumulation of nucleic acids.

Such a polymer includes chitin, a natural nitrogenous polysaccharide conferring white and hard structures that make up the exoskeleton of insects, crustaceans and some fungi (Kumar, 2000). Deacetylation of chitin produces a derivative known as chitosan that has been widely...
used for many applications (Paul, 2000; Muzzarelli & Muzzarelli, 2005; Dash et al., 2011). Chitosan is a semi synthetic amino polysaccharides of glucosamine and N-acetyl-d-glucosamine linked together by β (1, 4) glycosidic bond (Gan & Wang, 2007) that possess favourable biological characteristics such as biodegradability, biocompatibility, low toxicity, haemostatic, bacteriostatic, fungistatic, anti-carcinogenic and anti-cholesteremic properties. Chitosan has been developed as a drug delivery carrier by complexing chitosan nanoparticles (CNPs) for the delivery of particular drugs for cancer treatments (Bugnicourt & Ladavière, 2016). CNPs also exhibit great performance as a non-viral vector for gene delivery (Fan et al., 2012).

Various methods have been employed to synthesise CNPs such as emulsion cross linking (Akbuga & Durmaz, 1994), coacervation or precipitation (Mao et al., 2001), emulsion droplet coalescence (Tokomitsu et al., 1999), and ionic gelation (Fan et al., 2012). Ionic gelation was found to be the most favourable due to its safer and more robust route of synthesis (Fan et al., 2012). The process involves physical cross linking instead of chemical cross linking, which are often toxic to organisms (Fan et al., 2012). A spherical particle known as nanoparticles was produced after both oppositely charged species of chitosan and TPP forming a complex (Dash et al., 2011). This study was aimed to synthesise and characterise CNPs.

**MATERIALS AND METHODS**

**Synthesis of chitosan/ TPP nanoparticles**

Chitosan nanoparticles were prepared by ionic gelation routes, with slight modifications based on methods described by Calvo et al. (1997). To obtain the smallest nanoparticle size of chitosan, experimental optimisation was performed by changing the concentration of chitosan and TPP solutions at a 1:1 ratio (v/v) with different volumes of TPP solutions.

The optimisation procedures were performed by first dissolving the chitosan powder in 0.5% (v/v) acetic acid solution to a concentration of 0.1, 0.5 and 1.0 mg/mL at pH 5.0. Subsequently, the TPP solution was prepared by dissolving TPP powder to concentrations ranging from 0.1, 0.5 and 1.0 mg/mL in ultrapure water at pH 7.4. Both solutions were then stirred overnight at room temperature. The chitosan solution was then passed through a 0.45 µm (Millipore, USA) syringe filter. To produce the nanoparticle suspension, synthesis was done using a series of chitosan and TPP concentrations (0.1, 0.5 and 1.0 mg/mL) at a 1:1 concentration ratio by dropwise additions of TPP at different volumes (25, 50, 100, 200, 250 and 300 µL) into 2 mL chitosan solution at the rate of 20-40 drops per minutes (Muhammadpourdounighi et al., 2010). The suspensions were then subjected to further characterisation.

**Physicochemical characterisation of CNPs and pNZ: VP2IL15-CNPs**

The particle size distribution of all nanoparticle samples was measured using dynamic light scattering (DLS) on a High-Performance Particle Sizer 3000 (Malvern Instrument, UK). Approximately 100 µL of sample firstly was resuspended in 900 µL ultra-pure water before analysis. Particle size mean values were obtained from the triplicate analysis of three different batches. Morphological and particle sizes of nanoparticle samples were cross-analysed by electron microscopy using H-7100 TEM (Hitachi, Japan). Nanoparticles were dropped on a parafilm and a carbon coated grid (Agar Scientific, UK) was placed on the CNPs drop, left for 5 minutes before fixing in 2% phosphotungstic acid (PTA) (Sigma, USA) for 5 minutes. Excess liquid was then removed from the grid using a Whatman paper (GE Healthcare, UK) and dried in a desiccator before viewing. The infrared spectra of CNPs and pNZ: VP2IL15-CNPs were then recorded on a Perkin-Elmer FTIR spectrometer (SPECTRUM 1000).

**RESULTS**

**Synthesis of CNPs**

As an optimisation, three different concentrations of chitosan and TPP (0.1, 0.5 and 1.0 mg/mL) were prepared and both solutions were combined using 1:1 concentration ratio with a series of TPP volumes (25, 50, 100, 200, 250 and 300 µL) into 2
mL chitosan and z-average particle size were measured by DLS. The result (Figure 1) showed all the three different combinations of chitosan and TPP solutions resulted to chitosan with nanoparticle sizes ranging from 45 nm to less than 300 nm. Combination of chitosan and TPP at 1.0 mg/mL showed the nanoparticle sizes.

**Figure 1.** (a) Size of CNPs generated with combination of chitosan and TPP solutions at concentration of 0.1 mg/mL; (b) Size of CNPs generated with combinations of chitosan and TPP solutions at concentration of 0.5 mg/mL; (c) Size of CNPs generated with combinations of chitosan and TPP solutions at concentration of 1.0 mg/mL.

**Characterisation of CNPs by TEM**

The morphology of optimised CNPs was observed by TEM. Typical CNPs showed spherical and polydisperse nature as shown in Figure 2. The CNPs conferred a round shape and smooth surfaces with sizes ranging from 3.22 nm to 23.69 nm.

**DISCUSSION**

Chitosan nanoparticles have been extensively studied as a drug delivery carrier for various antibiotics, anti-hypersensitive agents, anticancer agents, proteins, peptide drugs, and vaccines (Kunjachan et al., 2014). Other types of biomolecules have been investigated to be developed as nanocarrier, such as micelles, polymeric nanoparticles, polymer-drug conjugates and liposomes. Results from optimised CNPs conditions indicated that increased chitosan and TPP concentrations resulted in a subsequent increase towards the size of the nanoparticles produced; indicating that formation of CNPs at different sizes were somewhat dependent on specific concentrations of chitosan and TPP. CNPs measured by both DLS (~50 nm) and TEM (3-23 nm). Different sizes of CNPs obtained between TEM and DLS was observed. The differences between DLS and TEM occur due to swelling of the CNPs in liquid media and DLS gives a larger nanoparticle size while TEM gives an actual diameter of nanoparticles in dry state. The part of aggregation of the chitosan/TPP nanoparticles is probably because that the
As hydrogen bonding interactions between chitosan nanoparticles gradually become dominant in the drying process of 5 nm could in fact make a huge difference on the properties and biological functions. This result corresponded with findings by Cavlo et al. (1997) showed that the CNPs were synthesised at specific concentration of chitosan and TPP. In this study, optimum size of CNPs was achieved at 1.0 mg/mL of chitosan and TPP.

A gradual decrease of nanoparticle size was obtained with increasing volumes of TPP added to the chitosan solution. Studies by Fan et al. (2012) suggested a similar finding, where nanoparticle size decreased with increasing volumes of TPP indicating that protonated amino group of chitosan was neutralized by TPP anions. The best performed combination of chitosan and TPP was at 1.0 mg/mL with addition of 300 µL of TPP. The size of CNPs was found to be less than 50 nm as elucidated by DLS and TEM analyses. Nanoparticle sizes obtained in this study corresponded well with previous study (Fan et al., 2012) which exhibited spherical shape with particle size ranges from 30-50 nm.

Similar result also obtained in previous studies performed by ionic gelation method (Vimal et al., 2012; Gan & Wang, 2007; Xu & Du, 2003). FTIR analysis of CNPs showed a peak at 1560 cm⁻¹ indicating the N-H bending vibration that form as a result of chitosan and TPP interaction. Such a peak occurrence was also reported in another study by Muhammadpourdounighi et al. (2010).

CONCLUSION

Prabha et al. (2002) predicted smaller nanoparticles are more efficient in delivering drugs into tissues or cells compared to larger nanoparticle sizes. In this study, we successfully synthesised small size of CNPs which could be a potential candidate for further applications.

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REFERENCES


Recombinant expression and purification of adenocarcinoma GPR161 receptor

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Abstract. Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer and very few therapeutic options are currently available for its treatment. Interestingly, G-protein coupled receptor 161 (GPR161) is expressed in TNBC cells and can activate the mammalian target of the rapamycin complex 1 signaling pathway. GPR161 and Ras GTPase-activating-like protein, a protein involved in intracellular signaling, proliferation, and cellular adhesion, have been shown to genetically interact in human breast cancer cells. Targeting of GPR161 by monoclonal antibodies may therefore be a strategy to develop diagnostics and therapeutics for TNBC. Thus, to obtain such monoclonal antibodies, we synthesized the GPR161 gene de novo, cloned it into the pET32 expression plasmid, and used the recombinant plasmid to transform the competent BL21 (DE3) strain of Escherichia coli. The recombinant GPR161 gene was designed to contain an N-terminal thioredoxin tag, a thrombin site, the GPR161 sequence, and a C-terminal hexa-histidine tag to facilitate purification by metal-affinity chromatography. Following purification of the recombinant GPR161 (rGPR161) protein using a HisTrap column, we characterized the protein by Western blotting and mass spectrometry. The rGPR161 protein had a molecular mass of ~49 kDa and its identity as rGPR161 was confirmed by mass spectrometry data using the SwissProt database and the Mascot program. Future studies will involve the development of monoclonal antibodies using rGPR161 as the immunogen.

Keywords: Breast cancer, GPR161 receptor, monoclonal antibodies, recombinant protein, tumor diagnostics

INTRODUCTION

Triple-negative breast cancer (TNBC) accounts for approximately 15% cancer of the breast diagnosis in women and does not currently have effective targeted therapy. TNBC cells don’t express the estrogen (ER), progesterone (PR) and human epidermal growth factor 2 receptor (HER2), and are associated with a poor prognosis. The lack of ER, PR, and HER2 receptors on TNBC tumor cells restricts the use of hormone-based drugs for treatment. However, in breast cancer, there may be different types of cancer that meet the definition of TNBC, for example, basal-like breast cancers. This type of breast cancer is characterized by the absence or low levels of expression of ER and low levels of expression of HER2. Both types of cancers (TNBC and basal-like breast cancers) are characterized by the presence of a mutation in the BRCA1 gene and a high histological grade (Foulkes et al., 2010; Lachapelle and Foulkes, 2011; Rakha and Reis-Filho, 2009).

Breast cancer is generally highly...
heterogeneous and the different types are identified by their histopathological features, genetic alterations, and gene-expression profiles (Sotiriou and Pusztai, 2009). Kreike et al. (2007) characterized TNBC at the gene expression and histopathological levels. Based on their gene expression profiling study, they found that TNBC is itself heterogeneous and can be subdivided into at least five distinct subgroups. Because of the high levels of genetic heterogeneity, TNBC lacks both effective targeted therapies and diagnostics.

An important goal in studying triple-negative tumors is to identify novel therapeutic and diagnostic targets within this group of tumors. This is especially important as these tumors do not respond to ER- and HER2-targeted therapies since 73% of TNBC tumors are epidermal growth factor receptor (EGFR)-negative. Haffty et al. (2006) used simple commonly available markers for ER, PR, and HER2/neu and found that patients with TNBC have a relatively poor prognosis. Since they lack ER, PR, and HER2/neu receptors, patients with triple-negative breast cancer are not candidates for adjuvant hormonal therapy or trastuzumab.

G protein-coupled receptors (GPCRs) mediate different physiological processes and introduce the targets for a broad array of drugs for different diseases. GPCRs transduce extracellular signals from a variety of ligands through the activation of heterotrimeric G-proteins and downstream second messengers. Through this mechanism, rapid signaling events occur via the formation of short-lived secondary signals. GPCRs can also act as regulators of oncogenesis. Transactivation of signaling molecules, including EGFR, occurs by this mechanism. Because they can be activated by a wide variety of ligands, GPCRs play important roles in the pathogenesis of hormone-responsive tumors. For example, somatic mutations found in the thyrotropin receptor result in the activation of adenyl cyclase, leading to hyperactivation of thyroid adenomas. Thus, initiating mutations have been found in the luteinizing hormone receptor in Leydig-cell testicular tumors. Some new studies have also demonstrated the role of GPCRs in regulating cancer cell invasion and metastasis (Feigin, 2013).

Using large-scale genomic analysis, Feigin et al. (2014) discovered a poorly characterized receptor referred to as GPR161 that was up-regulated in TNBC. The GPR161 receptor was characterized as a prognostic biomarker for TNBC and was found to regulate the proliferation and migration of breast cancer cells. The authors also demonstrated the importance of GPR161 in the pathogenesis of TNBC by providing evidence that GPR161 promotes proliferation by activating mTORC1. The GPR161 receptor also regulates migration and invasion by disrupting the localization of E-cadherin (E-cad). The authors also demonstrated that GPR161 induces proliferation and migration in an IQGAP1-dependent manner. On the basis of these data, GPR161 appears to promote cancer cell proliferation and migration and is a promising drug target for the treatment of TNBC.

**MATERIALS AND METHODS**

**Bacterial strain, plasmids, and antibodies**

*E. coli* DH5α and BL21 (DE3) (Novagen, Madison, WI, USA), and plasmids pET28 and pET32 (Novagen) were used in this study. All *E. coli* strains were cultured in lysogeny broth (LB) medium. An anti-His-tag mouse monoclonal antibody (Sigma-Aldrich, Taufkirchen, Germany) and a peroxidase-conjugated secondary antibody (Sigma-Aldrich) were used for Western blotting.

**Gene synthesis**

The amino acid sequence of the GPR161 receptor isoform 1 (*Homo sapiens*) was extracted from PubMed with the NCBI sequence reference NP_001254538.1. Part of the sequence of the 7tm_GPCR (seven-transmembrane GPCR) encompassing amino acids 64-344 was selected for further study. The gene sequence was codon-optimized for expression in *E. coli* using Vector NTI 11.5 software, and the GPR161 receptor cDNA was synthesized by Macrogen Inc., Korea. The synthetic GPR161 cDNA was cloned into pET28 and pET32 plasmids using the NcoI and XhoI restriction sites so that the final pET32 plasmid construct contained an N-terminal thioredoxin tag, a thrombin site, the GPR161 receptor, and six C-terminal His-tags. The final pET28-based construct contained the same sequences except for the N-terminal thioredoxin.
Recombinant expression of GPR161 receptor

The predicted molecular weights of the His-tagged recombinant GPR161 in pET32 and pET28 were ~49 kDa and ~36 kDa, respectively.

Transformation of E. coli and expression of rGPR161

Competent BL21 (DE3) E. coli were transformed with the pET28 and pET32 plasmid vectors, with or without the gene insert, by electroporation using a MicroPulser (Bio-Rad, Hercules, CA, USA) under the following conditions: 100 ng of plasmid per 50 μL of cell suspension, at 2.5 kV, 25 μF, and 200 Ω. Electroporation duration was 5.2 ms. The transformed cells were incubated in 950 μL of superoptimal broth at 37°C for 1 h with rotary shaking at 200 rpm. Following this, 50 μL of cells were seeded onto LB agar containing ampicillin as the selection antibiotic and grown at 37°C for 16 h. Single colonies of transformants were cultured in 2 × YT broth containing ampicillin. In the middle of the logarithmic growth phase of the bacterial mass (OD600 = 0.6), 0.1 mmol/L of the inducer, isopropyl-β-D-1-galactopyranoside (IPTG), was added and the culture incubated for 6 h at 26°C. Cells were then pelleted by centrifugation at 6,000 × g for 7 min at 4°C. For sequencing, the GPR161 gene product was transformed into the DH5α E. coli strain. E. coli colonies were grown on solid agar medium and analyzed by PCR using Taq polymerase and M13 primers. Four positive clones were used for DNA purification and sequencing using the BigDye Terminator reagent kit (Thermo Fisher Scientific, Austin, TX, USA).

Cell lysis and chromatographic purification

For purification of the recombinant receptor, we used a protocol based on that reported by Attrill et al. (2009). Cells were lysed in an ice-cold buffer (20 mmol/L NaCl, 20 mmol/L HEPES, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.5% CHAPS, pH 7.5) using a UP200S ultrasonic disintegrator set at 24 kHz in a pulsating mode (10 pulses, 10 s per pulse). Cell debris was pelleted at 60,000 × g for 1 h. The detergent-solubilized fraction was filtered through a 0.2-μm syringe filter (Millipore, Burlington, MA, USA). Recombinant protein was purified using metal-chelate chromatography on Ni²⁺ ions using a 1-mL HisTrapTM HP column (GE Healthcare, Piscataway, NJ, USA).

The protein solution was placed onto the Ni²⁺-NTA column (2 mL bed volume) equilibrated with the same buffer. The column was washed with 10-bed volumes of the equilibration buffer (20 mmol/L Tris-HCl, pH 8.0, containing 500 mmol/L NaCl, 50 mmol/L imidazole, 0.5% CHAPS, and 0.1 PMSF). The GPR161 protein was then eluted with a 20–500 mmol/L linear imidazole gradient. The protein concentrations in the cell lysate and purified fractions were determined using the Bradford assay with bovine serum albumin as the standard (Bradford, 1976).

Western blotting

Fractions containing the GPR161 receptor were separated by electrophoresis on 11% polyacrylamide gels containing sodium dodecyl sulfate (SDS) according to the Laemmli method using a Bio-Rad electrophoresis apparatus (Bio-Rad) (Laemmli, 1970). Proteins were electrophoretically transferred onto nitrocellulose membranes using an immunoblotting device (Bio-Rad) according to a previously published method (Towbin et al., 1979).

For immunochemical detection, the nitrocellulose membranes were first incubated in a blocking solution (phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin) overnight at 4°C and then washed three times in PBS, pH 7.4, containing 0.05% Tween-20 (PBST). Membranes were incubated for 1.5 h at 37°C in a blocking buffer containing a 1:2000 dilution of a mouse monoclonal antibody against the His-tag. Subsequently, the membranes were rewashed as above and incubated in a blocking buffer with peroxidase-conjugated secondary antibody at 1:10000 dilution for 1 h at 37°C. The substrate solution was prepared immediately before use as follows: 0.01 g of 4-chloro-naphthol (Sigma, St. Louis, MO, USA) was dissolved in 2 mL of methanol, and mixed with 18 mL of buffer 1; finally, 0.01 mL of 3% (v/v) hydrogen peroxide was added. This substrate solution was applied to the blots for visualization of immunoreactive protein bands. The blot was placed in the substrate solution and incubated for 15 min at room temperature until the stained bands appeared.
Nano liquid chromatography and tandem mass spectrometry (nanoLC-MS/MS)

Workbench surfaces were routinely cleaned of dust using a clean, damp paper towel to avoid sample contamination with keratin. To ensure sample purity, we also used keratin-free Eppendorf tubes and barrier tips during sample preparation. Purified GPR161 samples were fractionated by 11% SDS-polyacrylamide gel electrophoresis (PAGE) and the gels were stained with Coomassie blue. The protein bands were precisely excised and transferred to keratin-free Eppendorf tubes. The excised bands were then divided into small gel fragments of 1 × 1 mm dimension. To destain the gel pieces of Coomassie blue, 100 μL of 100 mM ammonium bicarbonate in acetonitrile (1:1) was added and the gel fragments were incubated at 37°C for 30–40 min. After removing the supernatant, 5 mM dithiothreitol (DTT) was added to each tube and incubated at 60°C for 10 min. The DTT solution was then removed and 100 μL of 100 mM iodoacetamide was added and incubated at 37°C for 15 min to alkylate the reduced cysteine residues in the proteins. Excess reagent was then removed, and the gel pieces were washed twice in 100 μL of 50 mM ammonium bicarbonate. To remove any residual iodoacetamide, the gel pieces were subjected to two cycles of dehydration in 200 μL of 100% acetonitrile and rehydrated in 50 mM ammonium bicarbonate in water. The gel pieces were then dehydrated in 100% acetonitrile for 3–5 min to reduce their size, after which the acetonitrile was removed and the tubes dried for 5 min. Finally, 2 μL of 100 ng/μL trypsin and 50 μL of 50 mM ammonium bicarbonate were added, and the tubes were incubated overnight at 37°C to allow trypsin digestion to occur. On the following day, the supernatant containing the peptide mixtures resulting from trypsin digestion were transferred to clean Eppendorf tubes. For the second extraction of the digested peptides, the remaining gel pieces were washed in 50 μL of 50 mM ammonium bicarbonate, incubated for 15–20 min, and the supernatant transferred into the same tube containing the peptide mixture from the previous extraction. The contents of the tubes were dried using a vacuum concentrator at 45°C for 30-60 min. After complete removal of water, the resultant residue (containing the tryptic peptides) was dissolved in 10 μL of 0.1% trifluoroacetic acid, and the soluble peptide mixture was desalted using a Zip-tip kit (Millipore ZiptipsMicro-C18, 0.2 μL, Sigma).

The resulting mixture of tryptic peptides was separated using high-performance liquid chromatography (HPLC) and analyzed by in-line MS/MS. For LC-MS/MS, an Acclaim™ PepMap™ 100 C18 pre-column (5 mm × 300 cm; 5 μm particle size; Thermo Fisher Scientific) was used with a Dionex HPLC pump (Ultimate 3000 RSLC nano System, Thermo Fisher Scientific). The peptide mixture was separated on an Acclaim™ Pep-Map™ RSLC column (15 cm × 75 μm, 2 μm particle size; Thermo Fisher Scientific) using a 75-min multistage acetonitrile gradient (buffer A, 0.1% formic acid; buffer B, 90% acetonitrile/10% H₂O in 0.1% formic acid) at a flow rate of 0.3 μL/min. The gradient program for buffer B was as follows: 0 min - 2%, 10 min-2%, 58 min - 50%, 59 min - 99%, 69 min - 99%, 70 min - 2.0%, 75 min - 2.0%. The unmodified CaptiveSpray ion source (Capillary 1300 V, dry gas 3.0 L/min, dry temperature 150 °C) was used to interface the chromatography system with the Impact II (Bruker, Billerica, MA, USA). Subjecting the mixture of digested peptides to chromatography ensured the removal of low-molecular-weight impurities. The tandem MS/MS conditions were as follows: two of the most intense precursor ions to obtain sample data were selected for subsequent fragmentation with a full-time cycle of 3 s. The mass range was from 150 to 2,200 m/z under the positive ion mode.

The Mascot software was used to search the SwissProt 2016_10 database (552,884 sequences; 197,760,918 residues). Search parameters included variable modifications, including cysteine carbamidomethylation and methionine oxidation, a fragment ion mass tolerance of 0.6 Da, and a mass tolerance of the parent ion of 1.20 Da.

RESULTS

Design and construction of the expression vector

Comparison of the amino acid sequence of the GPR161 receptor isoform 1 with all GPR161 sequences present in the NCBI database revealed
that the GPR161 isoform 1 receptor is homologous to other members of this family of receptors (Figure 1). The codon-optimized nucleotide sequence of the selected region (length 897 bp including the restriction sites) (Figure 2) was inserted into the pET28 and pET32plasmids. Then, DH5α bacteria transformed with the vector containing the correct insert were identified and further confirmed by DNA sequencing to contain the insert encoding the fragment of recombinant transmembrane receptor GPR161 (rTMGPR161).

Figure 1. Homology of the GPR161 receptor isoform 1 with other members of the GPR161 family. Yellow color - the complete match of the nucleotide sequence of all receptor isoforms; blue and green colors - variations of nucleotide sequences.
Figure 2. Nucleotide sequence of the codon-optimized GPR161 fragment encoding amino acids 64-344 (rTMGPR161) (A), nucleotide sequence of the codon-optimized GPR161 fragment cloned into the pET32 plasmid (B) and nucleotide sequence of the codon-optimized GPR161 fragment cloned into the pET28 plasmid (C).
Transformation with the gene construct and establishment of an E. coli strain producing rTMGPR161

The expression vectors pET28/rTMGPR161 and pET32/rTMGPR161 were transformed into E. coli BL21. The strains obtained were then analyzed for the expression of the recombinant GPR161 protein. To detect protein expression, E. coli strains were cultured in LB medium supplemented with 0.2 mM IPTG at 26°C. After the addition of IPTG, samples of the E. coli culture were taken at different time points (2, 4, 6, and 24 h), sonicated, lysed, and subjected to SDS-PAGE followed by Coomassie blue staining. There was no recombinant protein expression in BL21 E. coli transformed with pET28/rTMGPR161(Figure 3(A)). In contrast, there was expression of rTMGPR161 in BL21 E. coli transformed with pET32/rTMGPR161 (Figure 3(B)). The rTMGPR161 protein was expressed 2 h after IPTG addition and had an approximate molecular mass of ~49 kDa.

![Figure 3](image-url)

**Figure 3.** SDS-PAGE of total proteins expressed in the strains BL21/pET28/rTMGPR161 (A) and BL21/pET32/rTMGPR161 (B). Lane 1 - E. coli culture without IPTG; Lane 2 - E. coli culture 2 h after IPTG addition; Lane 3 - E. coli culture 4 h after IPTG addition; Lane 4 - E. coli culture after 6 h incubation with IPTG; Lane 5 - E. coli culture after 24 h incubation with IPTG; MW - molecular weight markers.

Isolation and purification of rTMGPR161

A representative SDS-PAGE analysis of soluble and insoluble fractions from BL21/pET32/rTMGPR161 is shown in Figure 4. Following cell lysis and centrifugation to remove insoluble debris, the rTMGPR161 fusion protein remained present in the soluble fraction (Figure 4(A)) of the cell lysate, while it was not detectable in the pellet (Figure 4(B)).

To optimize the isolation and purification of rTMGPR161, the BL21/pET32/rTMGPR161 E. coli were cultured in 2 × YT medium containing ampicillin and various IPTG concentrations (0.05 mM, 0.1 mM, 0.2 mM, and 0.4 mM), at different time points (2, 4, 6, and 24 h) and at a temperature of 26°C. We found that the conditions for the optimal expression of rTMGPR161 were media containing 0.2 mM IPTG and 24 h incubation (Figure 5).

After purification of the rTMGPR161 protein by Ni²⁺-Sepharose chromatography and elution using a buffer containing 200 mM imidazole, we used SDS-PAGE and Coomassie blue staining to visualize the purified rTMGPR161 protein. As can be seen in Figure 6(A), chromatography yielded fraction containing purified rTMGPR161 with an expected molecular weight of about 49 kDa. To confirm expression of the correct protein, we used an anti-His-tag monoclonal antibody in Western blotting of the separated by SDS-PAGE rTMGPR161 samples (Figure 6(B)). This Western blotting analysis confirmed the presence of the hexahistidine tag on a protein with a molecular mass of ~49 kDa, which corresponds to the predicted molecular mass of rTMGPR161.

LC-MS/MS analysis of rTMGPR161

LC-MS/MS was used to confirm the identity of rTMGPR161. The MS/MS spectra of peaks corresponding to the fragmented ions of peptides derived from trypsin-digested rTMGPR161 were identified following SDS-PAGE, trypsin
digestion, and chromatographic separation. Trypsin-digested peptides are characterized by the presence of lysine or arginine residues at the C-terminus of the peptide. The MS/MS spectra were converted to mgf files using the DataAnalysis program. These files were submitted to the Mascot search engine, which compares the experimental data with theoretical mass spectra using available sequence databases of amino acids, such as NCBI or SwissProt.

The score (86.8) corresponded to only one protein, namely GPR161. Representative MS/MS spectra of the EGNLVIVVTLYKSYLLT LSNKF peptide of rGPR161 and its fragmentation ions are shown in Figure 7.

**Figure 4.** SDS-PAGE of supernatant (A) from lysates of E. coli BL21/pET32/rTMGPR161 and pellet (B). Lane 1 - E. coli culture without IPTG; Lane 2 - E. coli culture after 24 h incubation with IPTG; MW - molecular weight markers.

**Figure 5.** Graph of rTMGPR161 concentration versus IPTG concentration.
Recombinant expression of GPR161 receptor

Figure 6. SDS-PAGE (A) and Western blot (B) of purified proteins expressed and extracted from the BL21/pET32/rTMGPR161 E. coli. Lane 1 - purified rTMGPR161; MW – molecular weight markers.

Figure 7. MS/MS spectra of fragmented peptides derived from trypsin-digested rTMGPR161.

DISCUSSION

The GPR161 receptor is a prognostic biomarker of TNBC and an important regulator of the proliferation and migration of breast cancer cells. The GPR161 receptor decreases IQGAP1 serine phosphorylation and activates the mTOR/p70S6K signaling pathway. Genetic analyses of the GPR161 receptor in TNBC cells have identified two mutations. The first mutation (R91G) is located within the first extracellular loop and may, therefore, play a role in ligand coupling. The second mutation, S251G, is found within the third intracellular loop, a region which is phosphorylated in response to the activation of many GPCRs and is the site of interaction of β-arrestins (Feigin et al., 2014). Genetic analyses of other cancer types have uncovered their current amplification of the GPR161 gene in bladder urothelial carcinoma, lung adenocarcinoma, and melanoma. It is therefore likely that developing methods to target GPR161 may have diagnostic and therapeutic applications beyond breast cancer.

Electron microscopy research of this family of receptors has determined that these receptors include seven transmembrane α-helices.
Moreover, each transmembrane helix has several specific residues and a common three-dimensional structure that is conserved throughout all GPCRs (Lomize et al., 1999). The transmembrane protein structure contains seven stretches of 20–30 hydrophobic amino acids that form the membrane-spanning α-helices. The protein has an extracellular N-terminus and a cytoplasmic C-terminus. The N-termini of GPCRs varies greatly in length, ranging from seven amino acids for the adenosine receptor to over 300 amino acid residues for the glycoprotein hormone receptors. The C-terminus has one phosphorylation site, which can influence signal transduction across the membrane. Furthermore, the C-terminal cysteine may be palmitoylated, through building an additional cytoplasmic loop, which may affect receptor mobility or G-protein coupling (Probst et al., 1992).

The ligand-binding sites of GPCRs have been partly described through both biochemical and molecular biological methods. Helical amino acids have been classified as (A) amino acids that are in association with membrane lipids and (B) amino acids that are not in association with membrane lipids (Baldwin, 1993). Amino acid residues that are conserved within GPCRs contribute to the specificity of ligand binding. For example, two conserved serines in transmembrane helix 5 of the β 2-adrenergic receptor (Ser204 and Ser207) have been involved in building hydrogen bonds with the meta- and para-hydroxyl groups of adrenergic agonists. In many hormone GPCRs, the N-terminus is glycosylated and is rich in cysteine residues that may form disulfide bridges and help maintain the three-dimensional structure of the protein. However, in the absence of the extracellular N-terminus, ligands can still connect to the seven-transmembrane components of the receptor, albeit with a more inferior affinity (Ji and Ji, 1991). This suggests that GPCRs may include both a site extracellular binding with high affinity and a site in the within transmembrane domains of low affinity. It is possible that the site of the extracellular binding with high affinity created to capture the ligand and present it to the intramembranous binding pocket for signal transduction.

On the basis of this background, in the present study, we prepared the recombinant GPR161 receptor aiming to obtain monoclonal antibodies that can be used for developing diagnostics and therapeutics for TNBC. We generated a GPR161 fragment encompassing amino acids 64-344 (length 897 bp including restriction sites), cloned it into two different expression vectors, and transformed them into E. coli strain BL21 (DE3). We assessed and confirmed the expression of rTMGPR161 using one of the vectors, pET32/rTMGPR161, and optimized the conditions for the isolation and purification of rTMGPR161. Despite the many benefits of recombinant protein production in E. coli, this expression system has problems such as low expression levels or even lack of proteins expression (Soleimani et al., 2016). The lack of expression of rTMGPR161 in E. coli when using the pET28 vector is likely due to the formation of inclusion bodies. At the same time, the pET32 series is designed for cloning and high-level expression of peptide sequences fused with the thioredoxin protein. Thioredoxin significantly increases the solubility of proteins, while maintaining their biological activity (LaVallie et al., 1993).

We solubilized rTMGPR161 in a solubilization buffer and purified it by metal-affinity chromatography on a Ni²⁺-Sepharose column. As a result, a high initial concentration of the protein, which was effectively refolded while simultaneously being separated from high-molecular-weight protein aggregates, was obtained. Purification was achieved by spatially separating the rTMGPR161 molecules from each other through the pores of the column and a gradual increase in the concentration of imidazole. The LC-MS/MS analysis showed that rTMGPR161 was a fusion protein and contained thioredoxin and the TMGPR161 protein fragment.

The most widely used strategy to express GPCRs in E. coli is to use a fusion protein containing the receptor and a bacterial protein. The first example of this was the β 2-adrenergic receptor, which had the first 279 residues of β-galactosidase, a cytosolic protein naturally expressed in E. coli, fused to its N-terminus (Marullo et al., 1988). Fusion of the receptor to membrane proteins found in E. coli such as MBP or LamB increases the expression levels of the receptor by 10-fold. Nevertheless, identical expression levels of the receptor can be obtained
by using a strong IPTG-inducible promoter (gene 10 of the T7 bacteriophage) (Breyer et al., 1990; Nahmias et al., 1991). This difference clearly suggests that expression levels are receptor-dependent and that for each GPCR, a variety of genetic constructs must be regularly used because the results depend on the correct original sequence of the construct (Chapot et al., 1990).

CONCLUSION

In summary, we successfully expressed a recombinant form of GPR161, which we believe will be useful for the generation of either diagnostic or therapeutic monoclonal antibodies and can also be used as a protein therapeutic.

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REFERENCES


Dengue virus surveillance: circulation of DENV-1, DENV-2, DENV-3 and DENV-4 in mosquitoes around Taman Connaught, Cheras

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Abstract. Dengue, a mosquito-borne viral infection that causes dengue fever, hemorrhagic fever and dengue shock syndrome, is on a drastic rise in recent years. In Malaysia, the distribution of dengue viruses is determined based on localized studies, where these data are essential to predict the epidemiology of dengue infections. Therefore, a study was conducted in Taman Connaught, Cheras, determining dengue virus serotypes in field caught Aedes mosquitoes. Out of 1,147 mosquitoes captured from September to December 2017, 156 Aedes spp. mosquitoes were identified based on their physical features using a digital microscope. Total RNA was then extracted from the head and thorax of the mosquitoes and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with DENV-1, DENV-2, DENV-3 and DENV-4 primer sets, respectively. The results show that at least one DENV serotype was detected in 91% (n=142) out of 156 Aedes spp. mosquitoes. The predominant serotype detected was DENV-2 [47.44% (n=74)], followed by DENV-4 [8.97% (n=14)], DENV-3 [7.05% (n=11)] and DENV-1 [3.85% (n=6)], respectively. Co-circulation of DENV-2 and -4 was the predominant co-infection detected in Aedes spp. mosquitoes. We also present the first report of DENV-1, -2 and -4 co-infection in field-caught Aedes spp. Localized surveillance mechanisms based on serotype circulation in the mosquito vector can be an effective monitoring system for the government and public health sector in order to reduce the incidence of dengue hotspots.

Keywords: Aedes spp., dengue virus, RT-PCR, serotypes, surveillance

INTRODUCTION

The Aedes mosquito is the vector for the dengue virus, which is the etiological agent for dengue fever, hemorrhagic fever and dengue shock syndrome. Dengue is now the most epidemiologically-important viral mosquito-borne disease in the world, with a steady increase in the number of dengue cases globally (World Health Organization, 2019; Armstrong et al., 2003).

Four dengue virus serotypes, DENV-1, DENV-2, DENV-3 and DENV-4, found at different frequencies in different geographic regions, are associated with the epidemic potential of DENV (Quintero-Gil et al., 2014). DENV serotypes use the same putative receptors in the
mosquito midgut, where the susceptibility of DENV infection in mosquitoes can alter the accuracy of predictions on DENV transmission (Carrington et al., 2014). Some viral variants may have a strong fitness advantage, such as better ability to produce and or utilize genetically different polymerases or capsid proteins (Pepin et al., 2008). This intra-host competition within the mosquito vector can occur between different serotypes of the same virus (Quintero-Gil et al., 2014).

Virus surveillance is crucial in detecting and predicting infection outbreaks, where it is an important infection-control tool to understand spatial or temporal infection dynamics and evaluate control interventions (Pérez-Castro et al., 2016). Virus surveillance can also serve as an important mechanism in the identification of viruses with higher virulence in studies of natural viral evolution (Cologna et al., 2005).

In Malaysia, the Dengue Situation Update for July 2019 reported a half-year incidence of 62,421 cases as of 29th June 2019, which is nearly double the incidence reported for the same duration in 2018 (World Health Organization, 2019). The extraordinary rise of dengue cases from year to year has been reported in previous studies where records show a 160% increase in dengue cases reported within the Kuala Lumpur metropolitan area from 2015 to 2016. Of a total of 8664 dengue cases reported in 2016, the Cheras district contributed 20% of these dengue cases (Mahmud et al., 2018) in comparison to other urban districts within Kuala Lumpur. Cheras was previously reported with the highest number of dengue cases in Kuala Lumpur, with the prevalence of all four DENV serotypes (Chew et al., 2012).

Hence, data on vector carriage may be useful in developing effective intervention strategies in dengue hotspot areas. Thusly, we report the surveillance of dengue-infected mosquito populations around Taman Connaught in Cheras to identify dengue virus serotypes at risk for transmission from vectors to humans.

MATERIALS AND METHODS

Specimen collection and identification
Convenience sampling of 1,147 field-caught mosquitoes was carried out from September 2017 to December 2017 (4 months) within a one km radius sampling area in Taman Connaught, Cheras which covered commercial, residential and recreational facilities. Five commercial ultraviolet light-emitting mosquito traps were placed at sheltered strategic locations around the sampling area, along building corridors with a high frequency of daily human activity. Mosquitoes were continuously collected in the mornings between 8-10am throughout the 4 months, stored in sterile containers kept on ice, then transported to the laboratory for species identification on the same day. Mosquitoes were categorized into Aedes and non-Aedes mosquitoes using a digital microscope (Dino-Lite Edge, AnMo Electronics Corporation, Taiwan) at 80X magnification, where the physical morphology of mosquitoes was determined based on the guidelines of Walter Reed Biosystematics Unit, by accessing information available at http://www.wrbu.org/mqID/mq_gnra/aeedes.htm. Once identified, mosquitoes were stored at -80°C prior to RNA extraction.

RNA extraction and reverse transcription
Viral titers of dengue are high in the head, thorax (where the salivary glands are located) and midgut of the mosquito (Tsai et al., 2017), which provide reliable dengue positivity screening via molecular methods (Pankhong et al., 2002; Thavara et al., 2006). Thus, in the present study, the head and thorax of individual Aedes mosquitoes were dissected using forceps and scalpel under a digital microscope (Dino-Lite Edge, AnMo Electronics Corporation, Taiwan) and used for subsequent reverse-transcription Polymerase Chain Reaction (RT-PCR). RNA extraction was done for the head and thorax of individual mosquitoes using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. Reverse transcription was then carried out in 20 μL reactions. Briefly, 0.5 μg of total extracted RNA and 1 μL of 0.2 μg of 100 pmol random hexamers (Thermo Fisher Scientific, USA) were added to PCR tube and topped up
with 0.1% DEPC water. Subsequently, the mixture was added with 4 µL of 5X reaction buffer, 0.5 µL of 20 U/µL RNase inhibitor, 2 µL of 10mM dNTP, and 1 µL of 200 U MMLV-Reverse Transcriptase (all from Thermo Fisher Scientific, USA). The resulting reverse-transcribed cDNA was used for subsequent PCR confirmation of Aedes spp. and PCR screening of DENV serotypes 1 to 4.

**PCR confirmation of Aedes spp.**

PCR primers to amplify the consensus region of the small unit ribosomal RNA (18S rRNA) gene of Aedes spp (as in Table 1) were designed based on the GenBank IDs AY988440.1 (A. aegypti) and HQ010437 (A. albopictus), respectively. PCR was carried out in 25 µL reactions with 2.5 µL of 10X Standard Taq Reaction Buffer, 0.5 µL of 10 mM dNTP, 0.125 µL of 5000 U/mL Taq DNA polymerase (all from New England Biolabs, USA), 0.5 µL of each 10 µM forward and reverse primers (IDT, Singapore), and 1.0 µL of reverse-transcribed cDNA, and then topped up with nuclease-free water. The PCR reactions were subjected to the following cycling conditions in a thermal cycler (Nexus Gradient, Eppendorf, Germany): initial denaturation at 95ºC for 30 s; then 35 cycles of denaturation at 95ºC for 15 s, annealing at 53.3ºC for 30 s, and extension at 68ºC for 30 s; followed by a final extension at 68ºC for 5 min. PCR products were analyzed using 1% agarose gel electrophoresed in standard Tris Borate EDTA buffer at 80 V for 30 min, and visualized under ultraviolet light (Quantum ST4 Transilluminator + Quantum Capt Softwares, Viber Lourmat, France). Representative PCR products were gel-purified (GF-1 Gel DNA Recovery Kit, Vivantis Technologies Sdn. Bhd, Malaysia) for DNA sequencing (MyTACG, Taiwan) with the respective forward and reverse primers for PCR amplicon confirmation.

**PCR screening of DENV-1 to DENV-4**

PCR primers previously designed by Tanaka (1993) were used to detect DENV-1, DENV-2, DENV-3 and DENV-4 in Aedes spp.-positive samples. PCR was carried out using the cDNA of individual mosquitoes as before, with the exception of the specific DENV primers and the respective annealing temperature (Table 1). Every PCR run for a specific DENV included the respective DENV-specific positive control (a kind gift from Dr. Chee Hui Yee of Universiti Putra Malaysia), and a no-template control. DENV positive controls and representative were confirmed for primer specificity via DNA sequencing with the respective DENV primers.

### Table 1. All PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
<th>Optimized annealing temperature (°C)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
</table>
| Aedes spp. 18S rRNA | F: 5’-CATCGTGGTTGACTTCTGCT-3’
R: 5’-AAAGGGGAGGGACGTAATC-3’ | 53.3 | 262 |
| DENV-1 [16] | F: 5’-GGACTGGCTATGGAGTTTTG-3’
R: 5’-ATGGGTTGTGGCCTAATCAT-3’ | 49 | 490 |
| DENV-2 [16] | F: 5’-GGTTCCCTCTGGCAAAACACTCCA-3’
R: 5’-ATGGGTTGTGGCCTAATCAT-3’ | 45 | 230 |
| DENV-3 [16] | F: 5’-GGTGGCTTACACAGCCCTATTTG-3’
R: 5’-TGACATCTCCTCCAAGCGCCTG-3’ | 51.5 | 320 |
| DENV-4 [16] | F: 5’-CCATTATGGCTGTGTTTGTTTTG-3’
R: 5’-CTTCATCTCCTGCTTCTACTTCT-3’ | 46 | 398 |

**RESULTS**

Out of a total of 1,147 mosquitoes caught in this study, 156 were morphologically identified as Aedes spp., of which 74.7% were A. aegypti while 18.6% were A. albopictus.

The average concentration of the extracted RNA from the 156 Aedes spp. individuals was 90 ng/µL. Reverse transcription-PCR amplification
with 18S rRNA primers specific for *Aedes* spp. confirmed the morphological identification of all individuals as *Aedes* spp. (Figure 1).

Subsequent PCR with DENV-1 to DENV-4 primers specific to the respective dengue virus serotypes (Figures 2A and 2B) revealed that out of 156 *Aedes* spp., DENV-2 was the most prevalent single serotype detected [47.4%, n=74], followed by DENV-4 [9% (n=14)], DENV-3 [7.1% (n=11)] and DENV-1 [3.8% (n=6)], respectively (Table 2). Fourteen mosquitoes (9%) did not carry any of the four dengue virus serotypes.

Our results also revealed multiple dengue virus serotypes in 37 mosquitoes (23.7%), where DENV-2 + DENV-4 was the predominant co-infection detected (Table 3), occurring in 12 individuals. Co-detection of up to three dengue virus serotypes (DENVs-1, -2 and -4) was observed in four mosquitoes.

**Table 2.** *Aedes* spp. and DENV serotypes detected in this study. *Aedes aegypti* is the predominant *Aedes* spp. (74.4%), while DENV-2 is the predominant serotype (47.4%) in this study.

<table>
<thead>
<tr>
<th><em>Aedes</em> species</th>
<th>DENV detected in individual mosquitoes [%]</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DENV-1</td>
<td>DENV-2</td>
</tr>
<tr>
<td><em>A. albopictus</em></td>
<td>0 [0.0]</td>
<td>19 [12.2]</td>
</tr>
<tr>
<td>Other*</td>
<td>1 [0.6]</td>
<td>7 [4.5]</td>
</tr>
</tbody>
</table>

*Other *Aedes* spp.: *A. togoi* and *A. pseudoniveus*

^Refer to Table 3
Table 3. DENV serotypes co-detected in this study. DENV-2 + DENV-4 are the predominant co-detected serotypes (32.4%) in this study.

<table>
<thead>
<tr>
<th>DENV serotype</th>
<th>Mixed dengue serotypes detected in individual mosquitoes [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DENV-1</td>
</tr>
<tr>
<td>DENV-1</td>
<td>/</td>
</tr>
<tr>
<td>DENV-3</td>
<td>-</td>
</tr>
<tr>
<td>DENV-1 + DENV-2</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2B. Agarose gel electrophoresis image for DENV-1 to DENV-4 primer sets testing on representative mosquito. Lane 3 to 7, Lane 13 to 17, Lane 20 to 24, and Lane 30-34: PCR products of Mos 7, 9, 10, 11, and 12 tested by DENV-1 to DENV-4 primer sets, respectively. Lane 1, Lane 11, Lane 18 and Lane 28: negative controls. Lane 2, Lane 12, Lane 19, and Lane 29: positive controls, P1 to P4. Lane 9 and 26: 100bp DNA ladder marker (NEB, USA).

DISCUSSION

Of all 156 Aedes spp. mosquitoes caught, 142 tested positive for single or multiple dengue virus serotypes. The number of A. aegypti captured as compared to A. albopictus in this study is consistent with the general consensus that A. aegypti is gradually overtaking the native species of A. albopictus within the region (Rudnick, 1965). While A. aegypti is considered the primary vector for dengue, both species are significant vectors for arboviruses and are capable of carrying multiple arbovirus infections such as Chikungunya and Zika (Cogan, 2019).

A previous study in a hospital setting in Cheras found that although co-circulation of all four DENV serotypes were present, DENV-4 was the predominant serotype within 46 dengue positive cases with 97% being single or dual infection with DENV-4 (Chew et al., 2012). The present study, which is the first report of dengue virus serotypes in Aedes spp. in Cheras, found that DENV-2 (47.4%) was the predominant serotype in the vector instead, followed by DENV-4 (9%).

From general clinical data, there is observed seasonal drift in dominant dengue serotypes over the years with DENV-3 dominating in 2014 and DENV-4 gaining prominence in recent years, whilst still constituting less than 20% of all serotypes between 2000-2012 (Chew et al., 2012; Mohd-Zaki et al., 2014; Cogan, 2019). However, there has yet to be a clear correlation between the dominant DENV serotype found in infected humans and the dominant DENV serotype within mosquitoes, as infection dynamics depend
on the number of infected mosquitoes, proximity to humans, weather patterns, virological factors and other elements (Bar-Zeev et al., 1977; Cogan, 2019)

Co-infections are associated with more severe clinical manifestations of the disease, most notably with increased likelihood of developing Dengue Hemorrhagic Fever (DHF) (Gubler, 1998). The circulation of multiple DENV serotypes in mosquito vectors may increase both the occurrence of co-infection within humans and also the risk of subsequent secondary infection with a different dengue serotype, leading to poorer prognoses (Dhanoa et al., 2016). DENV-2 + DENV-3 co-infections in individual mosquitoes (10 mosquitoes with DENV-2 + DENV-3 of 15 total co-infections) were previously reported in Selangor (Lau et al., 2015).

In a 2016 study conducted in Johor Bahru (Malaysia) using 262 Dengue positive patient sera samples, 40 (15%) of samples were identified as co-infections (with DENV-1/DENV-2 contributing 85% of co-infections) (Dhanoa et al., 2016). The prevalence of several serotypes within a community or region collectively increases the impact of seasonal dengue outbreaks and co-infections and monitoring of dengue virus within the mosquito population and subsequent control measures may prove to be a vital precautionary step and serve as an early warning system to thwart the increasing burden of dengue outbreaks.

The present study found serotype co-infections in 37 individual Aedes spp. mosquitoes, with DENV-2 + DENV-4 as the prevalent co-infection. Interestingly, our study reports four individual Aedes spp. mosquitoes co-infected with three DENV serotypes (DENV-1, -2 and -4) which, to our knowledge, is the first such report of tri-DENV co-infections in field-caught Aedes spp.

In conclusion, this study suggests that DENV-2 is the predominant DENV serotype in the Taman Connaught area, with a large number of mosquitoes carrying co-infections of dengue serotypes. Data on localized DENV serotype circulation in mosquito vectors has the potential to develop an effective monitoring mechanism to the government, public health officials and the general public in order to develop better containment and dengue eradication strategies targeting the dengue mosquito vector.

**CONCLUSION**

A proteomic method based on 2-DE technique was developed for qualitative and quantitative analysis of HL-60 and CCRF-CEM. 5-Aza and TSA have great potential as anti-cancer effect in HL-60 and CCRF-CEM. Comparative analyses revealed that both epigenetic modification drugs induced different expressed proteins. The differentially expressed proteins discovered in this study may provide an overview to the protein alteration in a variety of cell functions and biological processes which could provide potential information in understanding the progression of leukemia. This preliminary finding is an early stage to provide fundamental information on disease mechanism and potential treatment.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


A study of the effect of Paclitaxel, Docetaxel and Tamoxifen citrate compounds found in *Taxus wallichiana* on the Beta-tubulin and Estrogen Receptor (ESR) proteins in humans using a bioinformatics approach

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Abstract. *Taxus wallichiana*, an indigenous plant of North East India contains bioactive compounds such as Tamoxifen citrate and taxol (paclitaxel and docetaxel) which inhibit the growth of cancerous cells. Tamoxifen citrate in its active form competitively inhibits the binding of estradiol or estrogen with estrogen receptor and results in tumor suppression whereas the taxol compounds stabilize the tubulin protein in microtubules and inhibit cell division in cancerous cells. In this approach, important domains and motifs of the protein receptors were studied and analyzed followed by homology modeling and its validation. Molecular docking studies were carried out via tubulin and estrogen receptor with paclitaxel, docetaxel, and endoxifen (an active form of tamoxifen citrate). Hence it was verified that tubulin and estrogen receptor contain binding sites for the bioactive compounds found in *T. wallichiana* and these bioactive compounds after binding with these receptors result in the prevention of the proliferation of the tumorous cells by stabilizing the microtubules and preventing the intake of steroidal hormones by cells respectively.

Keywords: Bioactive compounds, domains, homology modelling, molecular docking, taxol

INTRODUCTION

Taxol™, an anti-cancerous compound is present abundantly in conifer genera of *Taxus* and was first isolated from the bark of *Taxus brevifolia* (Pacific Yew) (Wani et al., 1971), in 1969 with a yield of 0.01%. The molecule was further investigated for its isolation from the wood and the needles of the plant and consequently, the first structure was reported in 1971 (Kingston, 2007; Kingston, 1990).

Before its importance as an anti-cancer compound, various studies have been reported on the bioactive compounds especially known as taxoids (Taxoid 2-alpha-hydroxylase, Taxoid 14-beta-hydroxylase) from the genus *Taxus* (Wani et al., 1971). One of the species of this genus, which is found specifically in the Himalayan region of India especially in the North-Eastern states, is *T. wallichiana* commonly known as East Himalayan Yew or Himalayan yew. This plant is declared as a threatened plant species by the International Union for Conservation of Nature (IUCN) (Thomas et al., 2011). This is an evergreen tree is found at altitudes between 1800 and 3300 m above mean sea level (MSL) in the temperate

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Himalayas and the hills of Meghalaya and Manipur at an altitude of 1500 m. This plant also contains another anti-cancerous compound called Tamoxifen citrate (Juyal et al., 2014; Sharma et al., 2015).

*T. wallichiana* is an ethnobotanically important species and has been used by the native for treating various ailments. Its uses have been described in traditional medicines such as Ayurveda and Unani. Along with anti-cancerous compounds it also contains compounds which are anti-convulsant, analgesic, antipyretic, anti-inflammatory, anti-bacterial, anti-fungal and many other properties (Juyal et al., 2014; Nissar et al., 2008; Khan et al., 2011; Qayum et al., 2012; Nissar et al., 2008).

The first evidence of taxol activity with responses in melanoma was reported in 1987, in ovarian cancer in 1989 and in breast cancer in 1991. Overview of the Taxol biosynthetic pathway is given in Figure 1. The boxed MEP (Methylerythritol Phosphate) Pathway is the plastidial route for the universal diterpenoid progenitor IPP and DMAPP precursor supply. The abbreviations are IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; IPPI, isopentenyl diphosphate isomerase; GGPPS, geranylgeranyl diphosphate synthase; TS, taxadiene synthase; and PAM, phenylalanine aminomutase. OPP denotes the diphosphate moiety; Ac and Bz denote acetyl and benzoyl groups, respectively. Because the relative order of C1 hydroxylation and oxetane formation is uncertain, the hypothetical intermediate illustrated could be at the level of an acylated hexanol rather than a heptanol bearing the C1 hydroxyl (Kovacs et al., 2007; Amos et al., 1999; Schiff et al., 1980).

Taxol and its semisynthetic analog docetaxel (Taxotere™) are now used (either as single agents or in combination with other drugs such as cisplatin) for the treatment of ovarian cancer, breast cancer, and non-small-cell lung cancer (Weirnik et al., 1987; McGuire et al., 1989; Holmes et al., 1991; Guiritte et al., 1986; Piccart et al., 2003; Ozols, 2003). Paclitaxel consists of an eight-member taxane ring with a four-member oxetane ring and a bulky ester side chain at C-13 that is necessary for antitumor activity (Thomas et al., 2011). The chemical formula of paclitaxel is C_{47}H_{53}O_{14} and its molecular weight is 853.9 g/mol. Docetaxel differs from paclitaxel in the 10-position on the baccatin ring and in the 3’-position of the lateral chain and has a chemical formula of C_{45}H_{59}NO_{14} and a molecular weight of 807.9 g/mol. The compounds found in *T. wallichiana* are Paclitaxel and Docetaxel (Davies et al., 2003), which bind at the taxane pocket of the beta chain of the tubulin protein, which in return stabilizes the microtubules during cell division. The disintegration of microtubules is required for the formation of two new daughter cells at the end of cell division, and thus stabilizing these microtubules prevents further cell division and hence prevents cancerous cells from dividing rapidly (Long et al., 1994; Sakkaraippan et al., 2005).

On the other hand, Tamoxifen citrate in its active form i.e endoxifen competitively binds with estrogen receptor α (ER-α) instead of estradiol and results in the lowering of cell division of the cells. Studies have shown that there is a direct connection between the increased responses of ER-α with most cases of cancer in mammary glands. Endoxifen thus blocks the working of ER-α and significantly lowers the rate of cancerous cell division (Fisher et al., 1989; Fisher et al., 1996; Yu et al., 2001; Rowinsky et al., 1995; Suffness et al., 1993). Biosynthesis of the anticancer drug Taxol in *Taxus* (yew) species involves 19 steps from the universal diterpenoid progenitor geranylgeranyl diphosphate (Croteau et al., 2006).

Beta-Tubulin is a subunit of tubulin. Tubulin is one of several members of a small family of globular proteins. It is the major constituent of microtubules. There are two of the most common members of the tubulin family: alpha-tubulin and beta-tubulin, and together their dimers form microtubules. The dimers of alpha- and beta-tubulin bind to GTP and assemble onto the (+) ends of microtubules while in the GTP-bound state. After the dimer is incorporated into the microtubule, the molecule of GTP bound to the beta-tubulin subunit eventually hydrolyzes into GDP through inter-dimer contacts along the microtubule protofilament. Beta-tubulin faces the plus end of the microtubule while alpha-tubulin faces the minus end (Heald et al., 2002; Howard et al., 2003). In the context of cancer, the tubulin family of proteins is recognized as the target of the tubulin-binding chemotherapeutics, which
suppresses the dynamics of the mitotic spindle to cause mitotic arrest and cell death. Also, changes in the microtubule stability and the expression of different tubulin isotypes as well as altered post-translational modifications have been stated for a variety of cancers (Amelia et al., 2014).

Figure 1. Overview of the Taxol biosynthetic pathway (Croteau et al., 2006).

MATERIALS AND METHOD

Data set
The taxol compounds, Paclitaxel and Docetaxel have PubChem CID 36314 and 148124 respectively. And Tamoxifen citrate along with its active form Endoxifen has PubChem CID 273525 and 10090750. These compounds were all retrieved from the PubChem compound (Wang et al., 2009). The FASTA sequence for Estrogen Receptor (ESR) and Beta-tubulin protein of Homo sapiens were retrieved from NCBI (National Center for Biotechnology Information) with accession number AAA52399.1 and AAB59507.1 respectively and Gene Information - 182193 and 338695 respectively.

Domain and motif analysis
After the sequences for the proteins Estrogen Receptor and Beta-tubulin were retrieved, their domain and motifs were analysed using cDART.
(Conserved Domain Architecture Retrieval Tool) and Motif Scan (Geer et al., 2002; Marchler et al., 2007). The domains and their superfamilies were accessed using NCBI Conserved Domain Database (Pagni et al., 2007).

**Homology modelling**
Using Swiss-Model server (Guex et al., 2009), protein homology modelling was carried out for both Estrogen Receptor (ESR) and Beta-tubulin after their FASTA sequences were retrieved from NCBI (Biasini et al., 2014; Bienert et al., 2017; Bertoni et al., 2007). And these structures were further validated using Ramachandran Plot via PROCHECK (Laskowski et al., 1993; Laskowski et al., 1996). The templates generated were 4ZNH and 5IJO as PDB IDs respectively which had the highest similarity percentage and were viewed using Chimera 1.11.2 (Pettersen et al., 2004).

**Molecular docking**
Molecular docking studies were carried out using the PATCHDOCK server (Duhovny et al., 2002; Schneiderman-Duhovny et al., 2005) and the energy minimization was carried out with QMEAN server (Benkert et al., 2009) and the molecular docking results were further refined with the help of FIREDOCK (Andruiser et al., 2007; Mashiach et al., 2008). The refined docked structures were viewed using Chimera 1.11.2 (Pettersen et al., 2004) and further analysis of the amino acids present in the active site was analyzed using Pymol (DeLano, 2002). Further analysis means the positions of the amino acids in the docking area in their respective chains. Energy minimization methods can precisely locate minimum confirmation by mathematically "homing in" on the energy function minima. The goal of energy minimization is to find a route from an initial confirmation to the nearest minimum-energy confirmation using the smallest number of calculations possible. Energy minimization can repair the distorted geometric by moving atoms to release internal constraints. The refinement of docked structures aims at side chain optimization. This method targets the problem of flexibility and scoring of solutions (docked structures).

**RESULTS**

**Domain and motif analysis**
After the analysis of the domains of the protein receptors, it is found that estrogen receptor has four domains of four different superfamilies whereas tubulin protein has only one domain. The four superfamilies of the estrogen receptor are Oestrogen receptor superfamily, DNA binding domain of nuclear receptors, ligand binding domain of nuclear receptors and oestrogen type nuclear receptor final C-terminal. The total architecture was found to be 265. These domains are found in the taxonomic spans of tetrapoda, gnathostomata, metazoan, bilateria, dipnoterapodomorpha and other taxonomic categories (Figure 2a). Tubulin protein has only one domain of tubulin superfamily and is found in euteleostomi, poecilia, eutheria, thamarchaeota, gossypium and in other cellular organisms. The total architecture is found to be 45 (Figure 2b). According to the motif analysis, the motifs of each protein are located along with the analysis of these proteins. Result of the motif analysis of Estrogen receptor, Ligand-binding domain of nuclear hormone receptor -pos: 351-543, raw-score =177.9, N-score =60.047, E-value =1.9e-53 (Figure 3a). (Oest_recep) Oestrogen receptor - pos: 1-181, raw-score = 464.0, N-score =144.636, E-value = 4.9e-138 (Figure 3b). (SapB_2) Saposin-like type B, region 2- pos: 527-547, raw-score = 9.5, N-score = 10.122, E-value = 0.0016 (Figure 3c). (zf-C4) Zinc finger, C4 type (two domains)- pos.: 183-258, raw-score = 178.2, N-score = 60.984, E-value = 2.2e-54 (Figure 3d). Results of the motif analysis of tubulin protein, Sigma-54 interaction domain (Sigma54_activat) - pos: 367-381, raw-score = 4.9, N-score = 9.735, E-value = 0.0039 (Figure 4a). Tubulin/FtsZ family, GTase domain - pos: 3-243, raw-score = 338.0, N-score = 111.096, E-value = 1.7e-104 (Figure 4b). Tubulin/FtsZ family, C-terminal domain- pos: 246-383, raw-score = 279.9, N-score = 89.212, E-value = 1.3e-82 (Figure 4c). Coagulation Factor V LSPD Repeat (LSPR) - pos: 284-292, raw-score = 1.4, N-score = 6.746, E-value = 3.8 (Figure 4d, Table 1).
Figure 2. (a) Domain analysis of Estrogen receptor shows 265 total architectures encompassing Oest_recep, NR|_DBD_like, NR_LBD, ESR1_C superfamily, (b) Domain analysis of Tubulin shows 45 total architecture encompassing Tubulin superfamily.
Figure 3. Motif analysis of tubulin protein (a) sigma – 54 interaction domain (b) GTPase domain (c) C-terminal domain and (d) Coagulation factor VLSPD Repeat (V LSPR), from investigating databases which includes HAMAP profiles [hamap], Pfam HMMs (local models) [pfam_fs], Pfam HMMs (global models) [pfam_ls].

Figure 4. Motif analysis of Estrogen receptor. (a) ligand binding domain of nuclear hormones (b) Oestrogen receptor (c) Saposin like type B (d) Zinc finger, from investigating databases which includes HAMAP profiles [hamap], Pfam HMMs (local models) [pfam_fs], Pfam HMMs (global models) [pfam_ls].
Table 1. Showing the motifs/domains present in the Estrogen Receptor and also in the Tubulin protein. It includes their position(s), raw score, N-score, and E-value.

<table>
<thead>
<tr>
<th>Motif analysis of the Estrogen Receptor</th>
<th>Position</th>
<th>Raw score</th>
<th>N score</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand binding domain of nuclear hormone receptor</td>
<td>351-543</td>
<td>177.9</td>
<td>60.047</td>
<td>1.9e-53</td>
</tr>
<tr>
<td>Oestrogen Receptor</td>
<td>1-181</td>
<td>464.0</td>
<td>144.636</td>
<td>4.9e-138</td>
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<tr>
<td>Saposin-like type B, region 2</td>
<td>527-547</td>
<td>9.5</td>
<td>10.122</td>
<td>0.0016</td>
</tr>
<tr>
<td>Zinc finger, C4 type domain</td>
<td>183-258</td>
<td>178.2</td>
<td>60.984</td>
<td>2.2e-54</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Motif analysis of Tubulin protein</th>
<th>Position</th>
<th>Raw score</th>
<th>N score</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma -54 interaction domain</td>
<td>367-381</td>
<td>4.9</td>
<td>9.735</td>
<td>0.0039</td>
</tr>
<tr>
<td>Tubulin/FtsZ family, GTPase domain</td>
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<td>338.0</td>
<td>111.096</td>
<td>1.7e-104</td>
</tr>
<tr>
<td>Tubulin/FtsZ family, C-terminal</td>
<td>246-383</td>
<td>279.9</td>
<td>89.212</td>
<td>1.3e-82</td>
</tr>
<tr>
<td>Coagulation Factor V, LSPD Repeat (LSPR)</td>
<td>284-292</td>
<td>1.4</td>
<td>6.746</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Homology modeling and validation
In the present work, the protein molecules i.e. Estrogen Receptor and Beta Tubulin were initially explored for the best homology modeling which determines the overall 3D structure of the amino acids present in the enzyme molecule. The homology modeling using the Swiss-Dock server depicted the best model of the enzyme molecule Estrogen Receptor (Figure 5a) and Beta Tubulin (Figure 6a). These structures were further validated by the corresponding Ramachandran Plot for Estrogen Receptor (Figure 5b) and Beta tubulin (Figure 6b) respectively. For Estrogen Receptor, the Ramachandran Plot depicted 1 of amino acids in the disallowed region with the maximum number of amino acid molecules in the favorable region. Most favorable regions showing 95.4% having 414 residues, additional allowed region showing 4.1% having 18 residues and generously allowed region showing 0.2% having only 1 residue. There was 1 residue found in the disallowed region. The Ramachandran Plot analysis further showed 856 as the total number of residues, the number of Glycine (Gly) and Proline (Pro) are 63 and 39, respectively, and the number of end residues (excluding Gly and Pro) was 4.

Computation of docking score between the ligands and receptor
Protein and other chemical molecule interaction outputs compute data which could be an advantage to understand the interaction between the ligands and the receptor. The ligands docetaxel, paclitaxel bind to β chain of tubulin protein and the ligand endoxifen (an active form of tamoxifen) binds to Estrogen Receptor α (ER α). We kept the clustering RMSD value restricted to 2 and the complex type was kept as the protein-small ligand. Figure 7, Table 2. The top 10 docking results are provided in a tabular format. Table 3.
Figure 5. (a) Three-dimensional structure of Estrogen Receptor modeled using the Swiss-Model server with its natural ligands. (b) Ramachandran Plot of 4ZNH.

Figure 6. (a) Three-dimensional structure of Beta Tubulin modeled using the Swiss-Model server with its natural ligands. (b) Ramachandran Plot of 5IJO.
Figure 7. Molecular docked structures of (a) Docetaxel and tubulin protein, (b) Paclitaxel and tubulin protein, (c) Endoxifen and estrogen receptor.
Table 2. Detailed docking analysis result showing interaction of receptors and ligands. The amino acid residue position of the receptors interacting with the ligands are also provided.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>β chain of tubulin</th>
<th>Estrogen Receptor α</th>
<th>Estrogen Receptor α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Docetaxel</td>
<td>Paclitaxel</td>
<td>Endoxifen</td>
</tr>
<tr>
<td><strong>Global Energy</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>-257.13</td>
<td>-275.19</td>
<td>-42.55</td>
</tr>
<tr>
<td>Score</td>
<td>-63.32</td>
<td>-56.47</td>
<td>-12.94</td>
</tr>
<tr>
<td>Area</td>
<td>312</td>
<td>306</td>
<td>3906</td>
</tr>
<tr>
<td><strong>Attractive VdW</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repulsive VdW</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positions (Residue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>39.50</td>
<td>41.80</td>
<td>518.10</td>
</tr>
<tr>
<td></td>
<td>-104.28</td>
<td>-123.78</td>
<td>-18.99</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>4.08</td>
</tr>
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</table>

Pro-32, Gly-34, Lys-58, Pro-80, Phe-81 Pro-272, Leu-289

Table 3. The top ten molecular docking results of Beta tubulin and Docetaxel, Beta Tubulin and Paclitaxel, and for Estrogen Receptor and Endoxifen.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Rank</th>
<th>Solution No.</th>
<th>Global Energy</th>
<th>Attractive Van der Waals</th>
<th>Repulsive Van der Waals</th>
<th>ACE</th>
<th>Amino acid position(s)</th>
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<tr>
<td>Beta tubulin</td>
<td>1</td>
<td>51</td>
<td>-257.13</td>
<td>-104.28</td>
<td>0</td>
<td>-63.32</td>
<td>Cys-12, Asp-177, Tyr-222, Thr-178, Ser-138, Asn-99, Ala-97, Gly-142, Gly-144, Thr-143, Gln-11, Gly-141, Asn-204, Val-169, Ile-16, Asn-226, Gln-15</td>
</tr>
<tr>
<td>and Docetaxel</td>
<td>2</td>
<td>56</td>
<td>-234.82</td>
<td>-92.24</td>
<td>0</td>
<td>-66.39</td>
<td>Glu-323, Pro-324, Pro-325, Ile-326, Leu-327, Glu-353, Ile-386, Leu-387, Gly-390, Trp-393, Arg-394, Gly-442</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15</td>
<td>-228.12</td>
<td>-102.25</td>
<td>0</td>
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<td></td>
<td>4</td>
<td>70</td>
<td>-227.39</td>
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<tr>
<td></td>
<td>5</td>
<td>8</td>
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<td>-101.82</td>
<td>18.48</td>
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<td>6</td>
<td>76</td>
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<tr>
<td></td>
<td>7</td>
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<tr>
<td></td>
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<td>-216.30</td>
<td>-91.40</td>
<td>0</td>
<td>-49.59</td>
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</tr>
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</table>

| and Paclitaxel  | 2    | 17           | -264.64       | -121.23                  | 31.02                   | -67.47 |                         |
|                 | 3    | 79           | -251.83       | -109.57                  | 0                       | -54.92 |                         |
|                 | 4    | 84           | -249.08       | -109.99                  | 0                       | -53.12 |                         |
|                 | 5    | 70           | -245.79       | -129.57                  | 0                       | -32.24 |                         |
|                 | 6    | 13           | -237.79       | -99.34                   | 0                       | -55.58 |                         |
|                 | 7    | 74           | -233.89       | -93.68                   | 0                       | -58.78 |                         |
|                 | 8    | 68           | -233.83       | -93.66                   | 0                       | -58.78 |                         |
|                 | 9    | 11           | -231.74       | -120.98                  | 34.75                   | -49.31 |                         |
|                 | 10   | 63           | -220.07       | -116.48                  | 0                       | -28.31 |                         |

|                                | 2    | 5            | -42.45         | -19.28                   | 6.23                    | -13.71 |                         |
|                                | 3    | 36           | -40.98         | -21.72                   | 6.88                    | -10.40 |                         |
|                                | 4    | 8            | -40.23         | -17.22                   | 6.92                    | -14.99 |                         |
|                                | 5    | 83           | -39.53         | -17.27                   | 6.02                    | -10.42 |                         |
|                                | 6    | 87           | -38.26         | -19.63                   | 5.08                    | -10.42 |                         |
|                                | 7    | 38           | -37.29         | -20.84                   | 10.63                   | -11.01 |                         |
|                                | 8    | 3            | -36.93         | -20.61                   | 7.77                    | -9.33 |                         |
|                                | 9    | 61           | -36.21         | -16.94                   | 7.69                    | -13.84 |                         |
|                                | 10   | 55           | -36.11         | -21.42                   | 9.93                    | -9.05 |                         |
DISCUSSION AND CONCLUSION

*Taxus wallichiana* is an ethnobotanical plant found in the Himalayan range of the Indian subcontinent and is traditionally parts of the plant that have been used to various ailments and it is found to have anti-convulsant, analgesic, antipyretic, anti-inflammatory, anti-allergic, antibiotic, anti-fungal and also anti-cancerous properties. The anti-cancerous properties are found to be due to the interaction of anti-cancerous compounds found specifically in the bark of the plant with proteins and as a result, controls the rapid growth of cancerous cells. Out of these compounds, Paclitaxel (Taxol®) and Docetaxel (Taxotere®) interact with tubulin protein and stabilizes the microtubules during cell division and prevents cell division. Whereas endoxifen, the active form tamoxifen binds with estrogen receptor and prevents uncontrolled cell division in steroidal cells. The ligands that are found in *Taxus wallichiana* are proved to have interacted with the receptors as a result of which it shows anti-cancerous properties. This plant is found in 1800 and 3300 m above mean sea level (MSL) in the temperate Himalayas and the hills of Meghalaya and Manipur at an altitude of 1500 m. According to the Forest & Environment Department, Government of Meghalaya, *Taxus wallichiana* is found in abundant in Mawphlang Sacred Groves of Meghalaya at latitude 25.449433707307875, longitude -91.75763019314581 and elevation is 1845 m. *Taxus wallichiana* is an endangered plant according to IUCN Red List (Thomas et al., 2011) and further care should be taken during the extraction of these anti-cancerous compounds.

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REFERENCES


Isolation and identification of the same type LMG1242 of biosurfactant-producing strain of Pseudomonas aeruginosa from different oil contaminated soils

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bIran Young Researchers and Elites Club

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Abstract. Emulsification is a major rate limiting step in any biological conversion of heavy hydrocarbons. Here, thirty bacterial strains were isolated from seven native consortia and screened for biosurfactant-producing activities which could enhance emulsification of heavy oils. The consortia were obtained by sampling from oil contaminated soils of different petroleum refineries of Iran. The oil spreading test, drop collapse test, emulsification index (E24) and surface tension measurements were used to evaluate the biosurfactant producing activities of the strains. A total number of 5 strains out of 30 were finally selected as the best biosurfactant-producing bacteria. The clear zone diameters in their oil spreading test were 4 to 5 cm, and E24 were 48 to 92%. The selected strains also properly lowered the surface tensions of the supernatants to 23.5-32 mN/m when grown on heavy diesel. All the selected strains were identified by 16S rRNA sequencing analysis as Pseudomonas aeruginosa LMG1242 (99.9% homology) which is a newly isolated type in oil polluted soils. The results suggest P. aeruginosa LMG1242 as a predominant and highly active biosurfactant producing bacterium which could be further evaluated in petroleum bioremediation and bioprocessing applications.

Keywords: P. aeruginosa, biosurfactant, emulsification, bioconversion, hydrocarbons, heavy oils

INTRODUCTION

Biosurfactans are mainly produced by microorganisms including different genera of bacteria and yeasts (Desai & Banat, 1997; Amaral et al., 2011). They belong to a structurally diverse group of surface active molecules which increase the surface area of hydrophobic substances, increase the bioavailability of hydrophobic substrates, and also regulate the adhesion of microorganisms to surfaces (Rosenberg & Ron, 1999). These molecules contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tensions between individual molecules at the surface and interface (Cooper, 1986).

The largest application of biosurfactant is the oil industry due to hydrophobicity of hydrocarbons (Bannat, 2000). They have been used in applications such as; enhanced oil recovery, oil spill bioremediation and removal of oil sludge from storage tanks (Mulligan, 2005). Crude oils and petroleum products contain thousands of hydrocarbons of different chain
lengths with low solubility in water. It is known that the availability of particular compounds is a major rate limiting factor in biodegradation rate of oils rather than the chemical structure of the compounds (Sugiura et al., 1997). Also, previous research mainly indicates that biosurfactants are more effective than chemical surfactants in enhancing the solubility and biodegradation rate of petroleum hydrocarbons (Chrzansowski et al., 2008; Cybulski et al., 2003; Souza et al., 2014). Biosurfactants increase the bioavailability of hydrocarbons by emulsification of the oil phase resulting in enhanced bacterial growth and bioconversion. This reveals the importance of identifying microorganisms responsible for biosurfactant production and emulsification of hydrocarbons in biological conversions.

Much research is conducted to identify biosurfactant-producing bacteria from different environments in contact with different hydrocarbon oils mainly for the purpose of bioremediation and biodegradation as well as enhanced oil recovery (Christova et al., 2019; Cai et al., 2017; Nwagumal et al., 2016; Souza et al., 2014; Bach & Gutnick, 2004). However, fewer reports are available on identification of biosurfactant-producing bacteria and their role in hydrocarbon bioprocessing applications such as viscosity reduction and heavy oil upgrading (Gudiña & Teixeira, 2017; Ceron-Camacho et al., 2013; Perfumo et al., 2010). Rocha et al. (2000) used Pseudomonas aeruginosa (USB-CS1) in making stabilized emulsions of heavy hydrocarbons getting proper viscosity reduction at ambient temperature. Viscosity reduction enables better properties for transportation and processing of the oils.

In our previous research, we obtained several microbial consortia from oil contaminated soils capable of bioconversion of heavy hydrocarbons (Ghavipanjeh et al., 2015; Ghollami et al., 2013; Ghavipanjeh et al., 2008). The consortia were able to increase the saturated fractions and decrease the resin fractions of the heavy oils thus improving the quality of the heavy oils. This happened through significant emulsification of the oils. However, the emulsification activities and consequently the performance of the consortia were sharply decreased after several passages and storage at -20°C. This revealed the loss of biosurfactant-producing bacteria or their activities upon preservation. It is expected that the addition of native strains to the consortia may stimulate biological conversion rates by enhancing the substrates availability to microorganisms and so lower mass transfer limitation (Abalos et al., 2004; Aparna et al., 2011). This could also impose a broader range of microorganisms to the consortia enabling them to convert a broader range of hydrocarbons (Barin et al., 2014).

The objective of this research was to isolate native biosurfactant-producing species which could be effectively used to facilitate the bioconversion rate of heavy hydrocarbons in bioremediation and bioconversion processes. Here, native bacterial strains were obtained from soil samples and adapted on heavy hydrocarbon oils. The consortia were screened for biosurfactant-producing properties by qualitative and quantitative methods. Pure bacterial strains were then isolated from the selected consortia and examined for biosurfactant-producing properties. The best isolates were then identified by 16S rRNA sequencing analysis.

**MATERIALS AND METHODS**

**Sampling**

Twelve soil samples were taken from highly aged oil spilled sites of different refineries of Iran including: Arak (AR1 to AR3 samples), Abadan (AH1 to AH3 samples), Shiraz (SH1 to SH3 samples) and Isfahan (IS1 to IS3 samples) refineries. The sampling was carried out in the spring 2016. The sampling locations are shown in Figure 1.

**Figure 1. Sampling locations.**
**Substrates and chemicals**

Heavy diesel and Isofeed were supplied by Isfahan refinery and used as the substrates to grow the consortia and the pure strains. Isofeed is a heavy end cut of vacuum distillation process in petroleum refineries. It is the feed going through Isomax unit process that is a catalytic cracking process for conversion of heavy hydrocarbons mainly to gasoline at a high pressure and temperature. Table 1 indicates the properties of the Isofeed and heavy diesel used for the experiments. The chemicals used for preparation of the culture media and experimental tests were of analytical grade produced by Merck.

**Table 1. Characteristics of Isofeed and heavy diesel from Isfahan refinery.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Isofeed</th>
<th>Heavy diesel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density at 15°C (g/cm³)</td>
<td>0.9</td>
<td>0.86</td>
</tr>
<tr>
<td>K. Viscosity (x 10^4 m²/s)</td>
<td>6.19 at 100°C</td>
<td>2.5 at 37.5°C</td>
</tr>
<tr>
<td>Flash point min. (°C)</td>
<td>210</td>
<td>64</td>
</tr>
<tr>
<td>Pour point max. (°C)</td>
<td>33</td>
<td>-3</td>
</tr>
<tr>
<td>Total sulfur content (wt%)</td>
<td>1.6</td>
<td>1</td>
</tr>
<tr>
<td>Distillation range (°C)</td>
<td>320-560</td>
<td>170-385</td>
</tr>
</tbody>
</table>

**Culture media**

A mineral salt medium (MSM) was used to cultivate the bacterial consortia and stains (Bushnell and Hass, 1941). The MSM medium contained (g/l): K₂HPO₄ (2.75), KH₂PO₄ (2.25), (NH₄)₂SO₄ (1), MgSO₄·7H₂O (0.2), NaCl (0.1), FeCl₃·6H₂O (0.02), and CaCl₂·2H₂O (0.01).

**Consortia enrichment**

In order to prepare the consortia, 5 gram of each soil sample was added into 50 ml MSM culture medium containing 5% v/v heavy diesel oil as the only carbon source. The pH was adjusted to 6.8. The cultures were kept on a shaking incubator at 160 rpm and 30°C for 4 days. At the end of this time, 5 ml of the aqueous phase was transferred into another flask containing MSM medium with the same amount of heavy diesel as the second enrichment culture. The samples were incubated once again at the previous conditions for 4 days. The subculturing procedure was repeated two more times. Each sample was prepared in triplicate to reduce the variation of results. The enrichment cultures were then kept in a refrigerator for the next experiments.

**Isolation of pure bacteria**

Bacterial isolations were made by streaking method on nutrient agar, MSM with a top layer of diesel oil, and by pore plate method. The morphologically distinct bacteria were selected for screening biosurfactant-producing bacteria. The isolates were cultured in nutrient broth medium and kept at 30°C and 160 rpm in a shaking incubator. The optical densities (OD) of the cultures were measured at wavelength of 660 nm. The cultures were used for inoculation into the MSM medium containing heavy diesel oil when OD₆₆₀ of the samples, reached to 1.

**Screening methods for detection of biosurfactant producers**

The consortia and the isolates were screened for biosurfactant-producing properties using the most common qualitative and quantitative methods (Bodour & Miller-Major, 1998; Youssef et al., 2004). The implemented test methods here are drop collapse test, oil spreading test, emulsification index and surface tension measurements.

**Drop collapse test**

For the drop collapse method, 2 μl of mineral oil was added to each well of a 96-well microtiter plate lid. The lid was equilibrated for 1 h at room temperature, and consequently 5 μl of the culture was delivered to center of the oil surface (Bodour & Miller-Maier, 1998). The shape of the drop on the surface of the oil was inspected after 1 min. Biosurfactant-producing cultures gave flat drops with scoring system ranging from ‘+’ to ‘++++’ corresponding from partial (poor) to complete (excellent) spreading on the oil surface. The sign ‘-’ indicates no spreading effect while complete spreading means high biosurfactant producing activity.

**Oil spreading method**

Distilled water (20 ml) was added to a Petri dish (10 cm diameter), followed by the addition of 10 μl of crude oil to the surface of water. A 10 μl of
biosurfactant solution was then added to the oil surface. The diameter of the clear zone formed on the oil surface was measured (Youssef et al., 2004).

**Emulsification index**
The cell-free culture broth was used to determine the emulsification capacity. 2 ml of cell-free supernatant was added to 2 ml of kerosene as the hydrophobic source and mixed in a vortex for 2 min. The mixture was stayed for 24 h and the height of emulsion layer was then measured. The emulsifying index (E24) was calculated as the percent of emulsion height to the total height of solution according to Cooper and Goldenberg (1987).

**Surface tension measurements**
The isolates were individually grown in heavy diesel and Isomax feed as the only carbon source, at 30°C and 160 rpm in a shaking incubator for one week. The cell-free supernatants were used to measure surface tensions by Kruss Tensiometer model K11. The measurements were made at 25°C and reported in mN/m (millinewton per meter).

**Bacterial identification method**
The selected biosurfactant-producing strains were identified by 16S rRNA sequencing analysis. The analysis was performed by ChromasPro software and the data was compared with EzBioCloud database.

**Experimental procedures**

**Screening of biosurfactant-producing consortia**
The prepared consortia (36 cultures, 3 from each soil sample) were cultured in MSM medium containing heavy diesel oil (5% v/v) and kept at 30°C and 160 rpm for one week. An identical flask was also prepared as the blank without any microorganism. The oil spreading test, drop collapse test and E24 test were implemented as initial tests on the supernatants in order to identify the biosurfactant-producing bacteria. The tests were implemented triplicate and presented as mean with standard deviations (±SD). The best performed strains were selected as the best biosurfactant-producing strains and then subjected to surface tension measurements and bacterial identification by 16S rRNA gene sequence analysis.

**RESULTS AND DISCUSSION**

**Screening of biosurfactant-producing consortia**
Based on the initial oil spreading and drop collapse tests on the 36 prepared consortia, 7 consortia signified most effective in biosurfactant-producing activities for which E24 tests were then implemented. Figure 2 shows the implemented oil spreading and E24 tests for AR2 consortium. As the figure shows the clear zone in oil spreading test can be observed as the empty interior circle. Also, the emulsified height can be observed in Figure 2-B as the upper layer (beneath a thin layer of oil on top) compared to the blank (Figure 2-A) in which the whole oil phase is on top.
The results of the implemented tests on the 7 selected consortia are indicated in Table 2. We know that the diameter of clear zone in oil spreading test is directly proportional to the concentration of biosurfactants in the medium (Morikawa, 2000). The diameter of clear zone for surfactin as an efficient biosurfactant was reported about 5.5 cm at concentration of 2000 mg/l (Youssef et al., 2004). As shown in Table 2, these values are about 6.5 to 7.8 cm for the selected consortia representing high surface activities of the consortia. The E24 tests are also greater than 50% indicating high emulsification activities. However, the drop collapse tests are well not consistent with the other two tests e.g. for IS3 in which the drop collapse test is poor but the others tests are quite good.

![Figure 2](image1.png)

**Figure 2.** Implemented oil spreading and E24 tests for AR2 consortium.

Table 2. The emulsification activities (E24) of the selected consortia (mean ± SD).

<table>
<thead>
<tr>
<th>Name of consortia</th>
<th>Oil spreading (cm)</th>
<th>Drop-collapse</th>
<th>E24 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH1</td>
<td>6.8± 0.65</td>
<td>+</td>
<td>50±1.4</td>
</tr>
<tr>
<td>AR1</td>
<td>7.8±0.76</td>
<td>+++</td>
<td>83±3.5</td>
</tr>
<tr>
<td>AR2</td>
<td>6.5±0.5</td>
<td>+++</td>
<td>75±2.1</td>
</tr>
<tr>
<td>IS2</td>
<td>7.3±0.29</td>
<td>++</td>
<td>50±1.5</td>
</tr>
<tr>
<td>IS3</td>
<td>7.3±0.29</td>
<td>+</td>
<td>66±1.8</td>
</tr>
<tr>
<td>SH1</td>
<td>7.0±0.5</td>
<td>++</td>
<td>75±2.2</td>
</tr>
<tr>
<td>SH2</td>
<td>7.8±0.58</td>
<td>+++</td>
<td>50±1.6</td>
</tr>
</tbody>
</table>

+ Poor
++ Good
+++ Very good

**Screening of biosurfactant-producing isolates**

A total of 30 morphologically different strains were isolated from the seven selected consortia. Table 3 shows the number and name of pure strains isolated from each consortium. The oil spreading test revealed 14 strains positive for biosurfactant productions which were then subjected to other tests. Table 4 shows the results of oil spreading, drop collapse and E24 tests for the 14 preferred strains. Some of the strains showed very little or no emulsification index and almost negative drop collapse test and weak oil spreading at the same time. Five strains which are highlighted in Table 4 exhibited better results compared to the others for at least two tests out of 3. The selected strains included AR1-A, AR2-B, SH1-A, SH2-A and SH2-C which were obtained from soil samples of Arak and Shiraz refineries. The clear zones for these strains are about 4 to 5 cm and E24 results are in the range of 48% to 92%. A comparison of the results with that of the consortia reveals reductions in oil spreading test while the emulsification activities (E24) are better for most of the selected strains.

Table 3. The numbers and names of bacterial strains isolated from the selected native consortia.

<table>
<thead>
<tr>
<th>Name of consortia</th>
<th>No. of isolated strains</th>
<th>Names of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR1</td>
<td>6</td>
<td>AR1-A, AR1-B, ... , AR1-F</td>
</tr>
<tr>
<td>AR2</td>
<td>6</td>
<td>AR2-A, AR2-B, ... , AR2-F</td>
</tr>
<tr>
<td>SH1</td>
<td>5</td>
<td>SH1-A, SH1-B, ... , SH1-F</td>
</tr>
<tr>
<td>SH2</td>
<td>4</td>
<td>SH2-A, SH2-B, SH2-C, SH2-D</td>
</tr>
<tr>
<td>IS2</td>
<td>2</td>
<td>IS2-A, IS2-B</td>
</tr>
<tr>
<td>IS3</td>
<td>3</td>
<td>IS3-A, IS3-B, IS3-C</td>
</tr>
<tr>
<td>AH1</td>
<td>4</td>
<td>AH1-A, AH1-B, AH1-C, AH1-D</td>
</tr>
</tbody>
</table>

Table 5 shows the surface tensions lowering results of the selected strains when grown in two different hydrocarbon substrates (Isofeed and heavy diesel). The surface tension of distilled water at 25℃ is about 72 mN/m. A good surfactant reduces the surface tension below 30 mN/m (Chrzanowski, 2008). The selected strains changed the surface to 23.5-32 mN/m when grown in heavy diesel and to 32.9-41.8 when grown in Isofeed. This indicates high biosurfactant-producing activities of the selected pure strains. The results are comparable with what obtained by Das and Mukherjee (2007) although, superior for AR2-B (23.5 mN/m surface tension).

Screening of same type LMG1242 P. aeruginosa from oil polluted sites

Table 5 also shows that the surface tensions of the supernatants are smaller when the bacteria grown on heavy diesel than that of Isofeed. This presumes the reduction of biosurfactant production due to reduction of bacterial activities in heavier hydrocarbons.

**Table 4.** The results of emulsification activity tests on the preferred isolates.

<table>
<thead>
<tr>
<th>Name of sample</th>
<th>Oil spreading test (cm)</th>
<th>Drop collapse test</th>
<th>E24 test (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR1-A</td>
<td>5±0.58</td>
<td>+++</td>
<td>48±1.5</td>
</tr>
<tr>
<td>AR1-B</td>
<td>2±0.58</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>AR2-A</td>
<td>5.5±0.50</td>
<td>++</td>
<td>32±2.4</td>
</tr>
<tr>
<td>AR2-B</td>
<td>4.5±0.43</td>
<td>+++</td>
<td>88.8±3.1</td>
</tr>
<tr>
<td>AR2-C</td>
<td>2.5±0.76</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>AR2-D</td>
<td>2±0.00</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>SH1-A</td>
<td>4±0.65</td>
<td>++++</td>
<td>89.6±2.8</td>
</tr>
<tr>
<td>SH1-B</td>
<td>4±0.00</td>
<td>-</td>
<td>48±2.6</td>
</tr>
<tr>
<td>SH2-A</td>
<td>5±0.67</td>
<td>+++</td>
<td>92±1.8</td>
</tr>
<tr>
<td>SH2-B</td>
<td>2±0.33</td>
<td>-</td>
<td>16±1.5</td>
</tr>
<tr>
<td>SH2-C</td>
<td>4.5±0.28</td>
<td>+++</td>
<td>56±1.2</td>
</tr>
<tr>
<td>IS2-A</td>
<td>2.5±0.29</td>
<td>-</td>
<td>8±1.0</td>
</tr>
<tr>
<td>IS3-A</td>
<td>3.5±0.76</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>AH1-A</td>
<td>3.5±0.29</td>
<td>++</td>
<td>48±1.8</td>
</tr>
</tbody>
</table>

- No activity
+ Poor
+++ Good
++++ Very good
+++++ Excellent

**Identification of the suitable species**

Figure 3 shows the phylogenetic tree of AR2-B strain. The phylogenetic trees of the other selected strains including; AR1-A, SH1-A, SH2-A and SH2-C (not shown here) were exactly the same as AR2-B. All the strains were identified as *P. aeruginosa* LMG1242 by 16S rRNA sequence analysis with 99.9% homology. Although, the visual and physical characteristics such color, emulsification tests and surface tension measurements of the strains were somehow different. It is known that the 16S rRNA gene sequence database may miss diversity in microbial products (e.g. biosurfactants, antibiotics) when identifying closely related isolates (Bodour et al., 2003). In addition, Choi et al. (2013) stated that the common molecular and serological methods may cause critical defects in the diagnosis and identification of *P. aeruginosa* strains. Yet, the results suggest *P. aeruginosa* LMG1242, as a predominant and active strain of bio-surfactant producing bacteria in the examined petroleum contaminated. *P. aeruginosa* species are greatly characterized for rhamnolipids production which exhibit surface actions (Sobri et al., 2018; Wasoh et al., 2017; Nordin et al., 2013; Rashedi et al., 2005). They are identified and investigated in many oil bioremediation activities indicating versatility and large capabilities of these species.

**Table 5.** The surface tensions measurements of selected isolates grown on heavy diesel and Isofeed.

<table>
<thead>
<tr>
<th>Name of isolate</th>
<th>Surface tension (mN/m, heavy diesel oil)</th>
<th>Surface tension (mN/m, Isofeed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR1-A</td>
<td>32±0.53</td>
<td>35.7±0.76</td>
</tr>
<tr>
<td>AR2-B</td>
<td>23.5±0.61</td>
<td>32.9±0.57</td>
</tr>
<tr>
<td>SH1-A</td>
<td>28.6±43</td>
<td>34±0.33</td>
</tr>
<tr>
<td>SH2-A</td>
<td>29±0.42</td>
<td>33±0.28</td>
</tr>
<tr>
<td>SH2-C</td>
<td>30.5±0.36</td>
<td>41.8±0.45</td>
</tr>
</tbody>
</table>

**Figure 3.** Phylogenetic tree of AR2-B strain.
CONCLUSION

Five bacterial strains were isolated from oil contaminated soils of different geographical locations of Iran and all identified as *Pseudomonas aeruginosa* LMG1242 by 16S rRNA sequence analysis although, they might be closely related strains. *Pseudomonas aeruginosa* LMG1242 is a newly isolated type detected in oil polluted soils and is the most active bacterium amongst other isolates in bioemulsification activities of heavy oils. The strains reduced the surface tension of the media well below 30 mN/m, even as low as 23.5 mN/m when grown in heavy diesel oil. However, their activities were somewhat reduced when grown in heavier oil (Isofeed). The results also showed that the biosurfactant-producing activities of the consortia were generally greater than that of the isolates. This implies the accumulative effects of the biosurfactant-producing bacteria in the consortium.

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REFERENCES


Aparna, A., Srinikethan, G., & Hegde, S. 2011. Screening of same type LMG1242 P. aeruginosa from oil polluted sites. *Amaral* P. F. F


Screening of same type LMG1242 P. aeruginosa from oil polluted sites


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- Suvik Assaw
- Tan Tian Tian
- Teoh Teow Chong
- Vicit Rizal Eh Suk
- Vipin Chandra Kalia
- Wan Mohd Aizat Wan Kamaruddin
- Wong Kok Kee
- Wong Pooi Fong
- Yam Hok Chai
- Yap Wai Sum
- Yap Wei Boon
- Yong Yean Kong
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