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Full Length Research Paper

Comparative biosorption of Pb^{2+} ions from aqueous solution using *Moringa oleifera* plant parts: Equilibrium, kinetics and thermodynamic studies

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This research article presents a comparative study of the adsorption properties of *Moringa oleifera* biomass using Pb^{2+} ions as test analyte. The investigated parameters which affected the adsorption process were the effect of pH, initial concentration, adsorbent dose, contact time, and temperature. The comparison of adsorption process was evaluated in the pH range of 3 to 8, concentration range from 5 to 20 ppm, temperature varied from 25 to 80°C, variation of contact time from 15 to 80 min, and dose of the adsorbent from 0.3 to 1.2 g. The results obtained showed that a high adsorbent dose is required for high adsorption capacity. The pH of 7 was most effective with temperature set at 25°C and contact time of 60 min. The Temkin, Dubunin-Radushkevich, Langmuir and Freundlich isotherms were applied and fitted well to the data and values of the parameters of these isotherm equations were calculated. The Langmuir isotherm proved to be the overall best isotherm. The adsorbent surface functional groups were identified with Fourier Transform Infrared (FTIR) spectroscopy. The maximum adsorption capacity obtained was 98% for seed, 96% for blended, 94% for pods, and 92% for leaves. Better thermodynamic and kinetic properties were obtained with the seeds and the blended samples. In total, these results indicate that the blended moringa biosobent can be employed as a low-cost biosorbent for the removal of lead ions from water.

Key words: Biosorption, *Moringa oleifera* biomass, adsorption isotherms, lead ions.

INTRODUCTION

Industrial activities and technological developments have led to the discharge of heavy metals into the environment. These heavy metals are toxic to humans and other forms of life because they are non-biodegradable hence accumulate in living organisms (Das et al., 2008; Kakalanga et al., 2012). Lead is among

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the most dangerous and most prevalent heavy metals from industrial processes (Kakalanga et al., 2012; Deng et al., 2008), which is extensively used in many important industrial applications such as battery manufacturing, printing, photographic materials, explosive manufacturing, coating, and steel industries (Kaya et al., 2009; Babarinde et al., 2012). Lead is toxic at low concentrations hence used as a test analyte in this study. According to World Health Organization (1984), permissible limit for lead in drinking water is 0.05 mg/L. Higher concentrations of lead ions in drinking water can damage nervous connections and cause blood and brain disorders, miscarriage, damage to sperm producing organs, and may result in death (Kaya et al., 2009; Khan et al., 2008; Igwe et al., 2006; Yakan et al., 2008; Memon et al., 2008; Adebayo et al., 2012). Thus, removal of toxic heavy metals such as lead is essential in environmental pollution control (El-Sayed et al., 2011). Conventional method for water treatment such as chemical precipitation, ion exchange and reverse osmosis are often costly and have inadequate efficiencies at low metal ion concentrations. Such procedures can generate toxic sludge hence the ultimate need for cheaper and effective procedures. Recently, the need for new technologies involving the removal of toxic metals from waste water has directed attention to biosorption due to low cost, free availability, high adsorption, possible reuse and flexible operation (Yang et al., 2009; Volesky, 2003; Park et al., 2010; Safa and Bhatti, 2011). *Moringa oleifera* husks have recently been used as biosorbents for determination of lead in chicken feed (Oliveira et al., 2017). However, a comparative analysis of different *M. oleifera* parts has not been done side-by-side.

Therefore, the purpose of this study was to investigate the possibility of the utilization of different parts of *M. oleifera* plant, and find which part has greater capacity for adsorption of Pb^{2+} ions as well as the effects of blending leaves, pods and seeds on biosorption using Pb^{2+} ions as the test analyte. *M. oleifera* biomass has been chosen since it has known medicinal properties hence the adsorbent will also have an antibacterial activity during the water treatment process. The biosorbent is envisaged to have both heavy metal adsorption and antimicrobial properties in the treatment of contaminated water. This report gives a comprehensive side by side adsorption characterization of *M. oleifera* parts and the blended composition for sorption of Pb^{2+} in water. The Temkin, Dubinin-Radushkevich, Freundlich and Langmuir isotherms have been used to describe the experimental equilibrium data.

MATERIALS AND METHODS

Equipment

The concentrations of Pb^{2+} in the solutions before and after biosorption were determined by FAAS (ThermoFisher AA-6800). The pH of the solution was measured using a pH meter (Orion Star A211) brined glass electrode. Fourier Transform Infrared (FT-IR) Spectrometer (Nicolet IS5) was used to identify the different functional groups present in the *M. oleifera* samples before and after removing Pb^{2+}. FTIR analyses were also used to determine the functional groups responsible for the Pb^{2+} binding with *M. oleifera* samples. The agitation was carried out by Reciprocated Shaker (HY-4).

Sample preparation

After sourcing, the seeds were removed from the fruit (called drumsticks). The seeds and pods were dried separately for about 2 weeks. The moringa leaves were also removed from the main plant and were placed at an open shelf in the laboratory for air drying for two weeks. The pods, seeds and leaves were ground separately to powder using a mechanical grinder. The powdered leaves, pods and seeds samples were sieved through 0.25 mm sieves. Blended *M. oleifera* sample (leaves, pods and seeds) was prepared by using a mixing ratio of 1:1:1 and mixed in a grinder for 2 min.

Biomass treatment

The method described by Mahmood et al. (2010) was modified and adopted in the treatment of the biosorbent. Each sample (8 g) weighed by analytical balance (GA110) was treated independently with 1000 ml, 0.1 M HCl with continuous stirring for 2 h to remove metals from the biosorbent and increase its surface area. It was washed with 500 ml deionized water; this was done three times, and then the sample was sundried for about 6 h. After the acid treatment, about 5 g of each sample was cleaned with 400 ml methanol to remove inorganic and organic matter from the sorbent surface. This was carried out for 2 h 30 min. The adsorbent pH was adjusted to 7 using 0.1 M NaOH, washed with deionized water, oven-dried for about 1 h. It was placed in an air-tight plastic container and put in a refrigerator at 4°C prior to analysis.

Solubility determination

Moringa samples (5.000 g) were weighed separately and transferred to a volumetric flask with 500 ml deionized water left stoppered for 24 h so as to dissolve all the soluble components (Qaiser et al., 2009). The mixture was filtered using vacuum filtration and the residue was collected, air dried for 3 h and oven dried at 65°C until a constant mass was obtained. Percentage solubility was calculated using Equation 1.

$$\% \text{ Solubility} = 100 (m1 - m2 / m1) \quad (1)$$

Where, *m1* and *m2* are masses of moringa samples before and after dissolution, respectively.

Characterization of biomass

The FTIR was used to determine the functional groups present in the moringa samples before the adsorption. Moringa samples were analyzed in the range 400 to 4000 cm^{-1}. KBr was used as background material.

Preparation of stock solutions and calibration standards

Stock solution of Pb^{2+} was prepared by dissolving 1.599 g of Pb $(NO_3)_2$ (Analytical grade, Sigma Aldrich) and making the solution to 1 L mark in a volumetric flask. The solutions contained 1.000

$\mu g/cm^3$ (1000 ppm) of Pb^{2+}. These solutions were used to prepare calibration standards of 5, 10, 15, 20, and 25 ppm for lead.

Effect of pH

The effect of pH on biosorption of Pb^{2+} ions was determined with the pH values 3, 4, 5, 6, 7, and 8 keeping the concentrations of metal ions at 20 ppm at room temperature (~25°C). The pH was adjusted using 0.1 M HCl and 0.1 M NaOH. 50 ml solutions of Pb^{2+} were transferred into conical flasks, 0.4 g of *M. oleifera* samples were added and the solution was shaken for 1 h at 150 rpm. The solutions were filtered and the filtrate was analyzed on the FAAS.

Effect of contact time

The effect of contact time on the process of biosorption was studied at the following time intervals 25, 40, 60, and 80 min at optimum pH of 7 at room temperature. 50 ml of 20 ppm Pb solution and 0.5 g of *M. oleifera* samples were transferred into conical flasks. The solutions were shaken at 150 rpm at different time intervals. The solutions were filtered and filtrate was analyzed on the FAAS.

Effect adsorbent dosage

To determine the effect of adsorbent dose, different amounts of 0.3, 0.6, 0.9 and 1.2 g of *M. oleifera* samples were suspended in 50 ml of 20 ppm Pb at room temperature. The solutions were transferred into conical flasks and left to shake at 150 rpm under optimum pH of 7 and contact time of 60 min. The solutions were filtered and the filtrate was analyzed on the FAAS.

Effect of initial ion concentration

The effect of initial concentration on the biosorption process was investigated under optimum conditions. 50 ml solutions of 5, 10, 15, and 20 ppm concentration of Pb were left to shake at 150 rpm at optimum pH of 7, contact time of 60 min, and biomass dosage of 0.4 g at room temperature. The solutions were filtered and the filtrate was analyzed on the FAAS.

Effect of temperature

The effect of temperature on the process of biosorption was studied at the following temperatures 25, 40, 60, and 80°C at the optimum pH of 7 and 50 ml of 20 ppm Pb and 0.5 g of moringa samples were transferred into conical flask. The solutions were shaken at 150 rpm at different temperatures. The solutions were filtered and filtrate was analyzed on the FAAS.

RESULTS AND DISCUSSION

Solubility determination

Solubility of *M. oleifera* samples was calculated using the Equation 1. The percentage of *M. oleifera* biomass recovery for different sample was 12.9% for leaves, 5.45% for pods, and 23.5% for seeds. There was a greater amount of sorbent components dissolved in the aqueous solution for all the sample sorbents and it does not participate in the sorption process.

FTIR analysis

The functional groups present in the moringa samples (pre and post Pb^{2+} adsorption) were determined using the FTIR spectroscopy. Figure 1 shows that the absorption at 3337.88 cm^{-1} in *M. oleifera* pods is due to OH stretching vibration (Kumar and Tamilarasan, 2013; Zhang et al., 2013; Sivaraj et al., 2001). Vibrations at 2916.32 cm^{-1} was due to CH stretching and 1732.08 cm^{-1} could be C=O (carbonyl) stretch (Lee et al., 2007). Vibrations at 1636.72 cm^{-1} can be ascribed to aromatic C=C bending or NH of an amide; 1238.72 and 1083.3 cm^{-1} were assigned to C-O group of an acid and an ester, respectively (Sharma and Uma Gode, 2010).

M. oleifera pods loaded with Pb^{2+} show the absorption peaks at 3337.88 cm^{-1} with the intensity of the peak shifted to 3311.29 cm^{-1}. This could be the stretching of the OH group due to the absorption. The disappearance of the sharp peak at 916.32 cm^{-1} could be the result of the C-H stretching involvement in absorptions. Absorption peak 1733.08 cm^{-1} lost intensity and shifted to 1730.08 cm^{-1} due to the stretch presence of C=O functional group of the carboxylic acids involvement in Pb^{2+} absorption. Repeated changes were also noted in the natural composition of *M. oleifera* pods which is the existence of peaks at wave number of 1238.72 and 1083.32 cm^{-1}. The repeated shift due to the C-O stretching suggests that C-O might be a functional group that Pb^{2+} can bind and react with it. The subsequence shift in the absorption peaks indicates the binding process on the surface of the *M. oleifera* pods.

Figure 2 shows absorption at 3282.84 cm^{-1} in blended *M. oleifera* sample due to OH stretching vibration (Kumar and Tamilarasan, 2013; Zhang et al., 2013; Sivaraj et al., 2001). The band at 2918.18 and 2853.36 cm^{-1} is due to C-H stretching. The absorption at 1743.18 cm^{-1} could be C=O (carbonyl) stretch and 1635.79 cm^{-1} is due to C=C stretching (Lee et al., 2007). The adsorption peaks at 1246.00 and 1017.90 cm^{-1} can be assigned to C-O group of an acid and an ester, respectively (Sharma and Uma Gode, 2010).

The blended *M. oleifera* loaded with Pb^{2+} shows the absorption peak at 3282.98 cm^{-1} lost intensity and shift to 3277.84 cm^{-1}. This could be the stretching of the OH group under the influence of adsorbed Pb^{2+}. It shows the absorption peaks at 2918.18 and 2853.36 cm^{-1} lost their intensity and shifted to 2914.08 and 2843.06 cm^{-1}, because of the C-H stretching. Absorption peak 1743.18 cm^{-1} lost intensity and shifted to 1732.78 cm^{-1}. The repeated shift due to the C-O and OH stretching suggests that both C-O and OH functional groups are involved in the binding or reacting with Pb^{2+}.

Figure 3 shows absorption at 3282.83 cm^{-1} in seeds is due to OH stretching vibration (Kumar and Tamilarasan, 2013; Zhang et al., 2013; Sivaraj et al., 2001). The band at 2918.13 and 2841.24 cm^{-1} is due to C-H stretching. The absorption at 1743.33 cm^{-1} could be C=O (carbonyl)

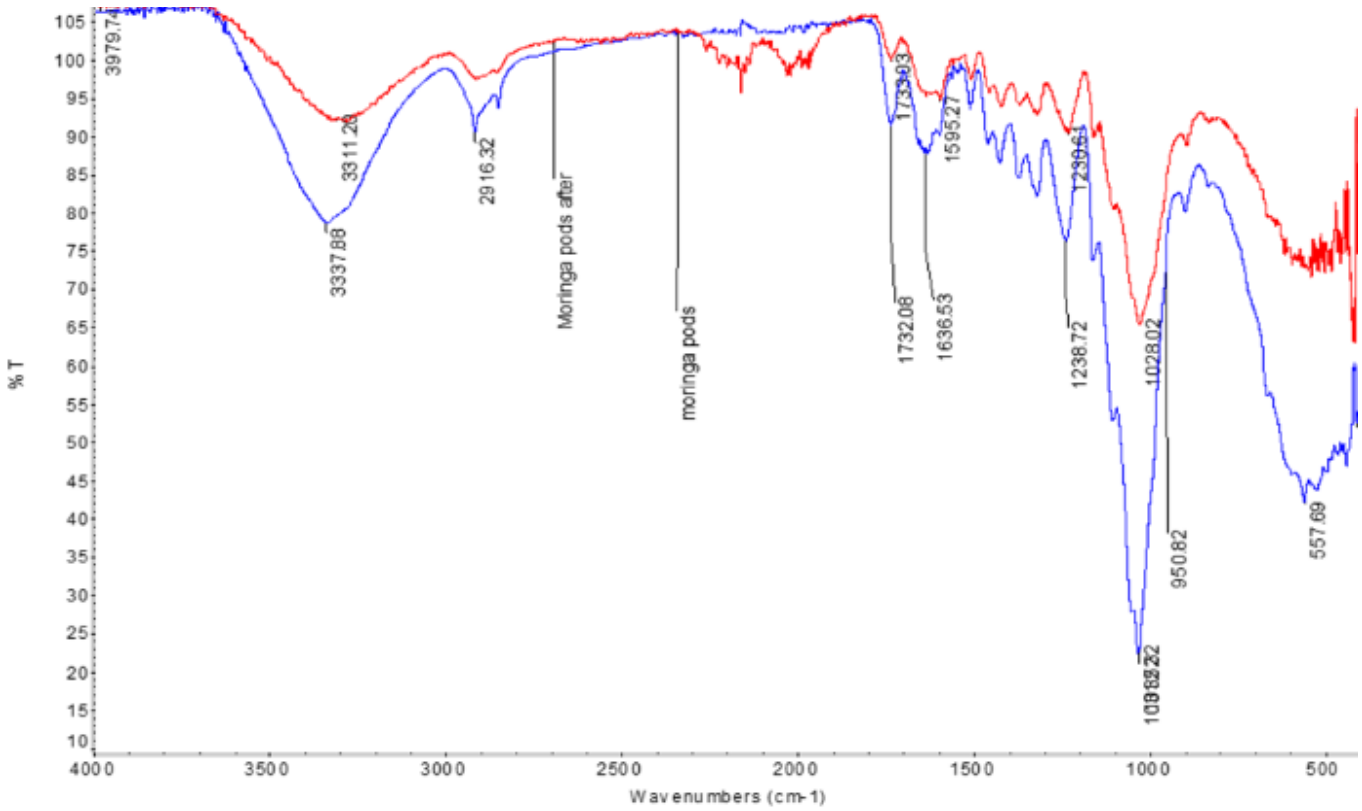

Figure 1. Pre (blue) and post (red) IR spectrum of pods.

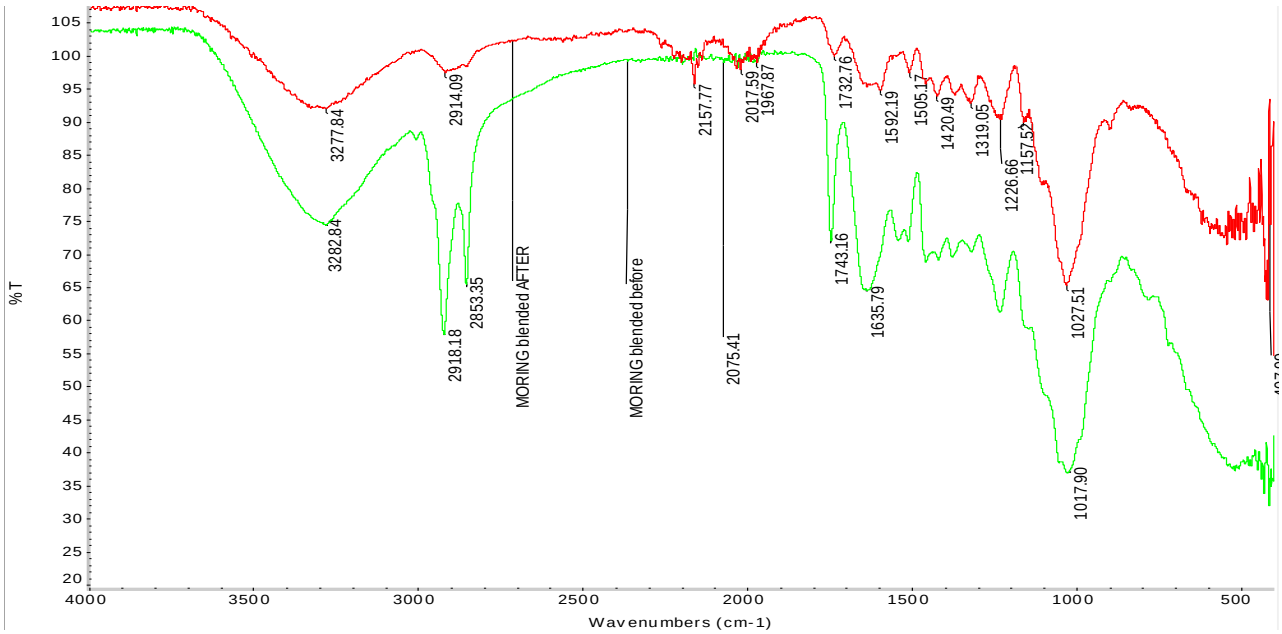

Figure 2. Pre (green) and post (red) IR spectra of the *Moringa oleifera* blended.

stretch and 1635.39 cm^{-1} is due to C=C stretching stretching (Lee et al., 2007).

M. oleifera seeds loaded with Pb^{2+} show the absorption peak at 3282.83 cm^{-1} lost intensity and shifted due to the stretching of the OH group. It shows the absorption peaks at 2918.13 cm^{-1} and 2841.24 cm^{-1} lost their intensity and

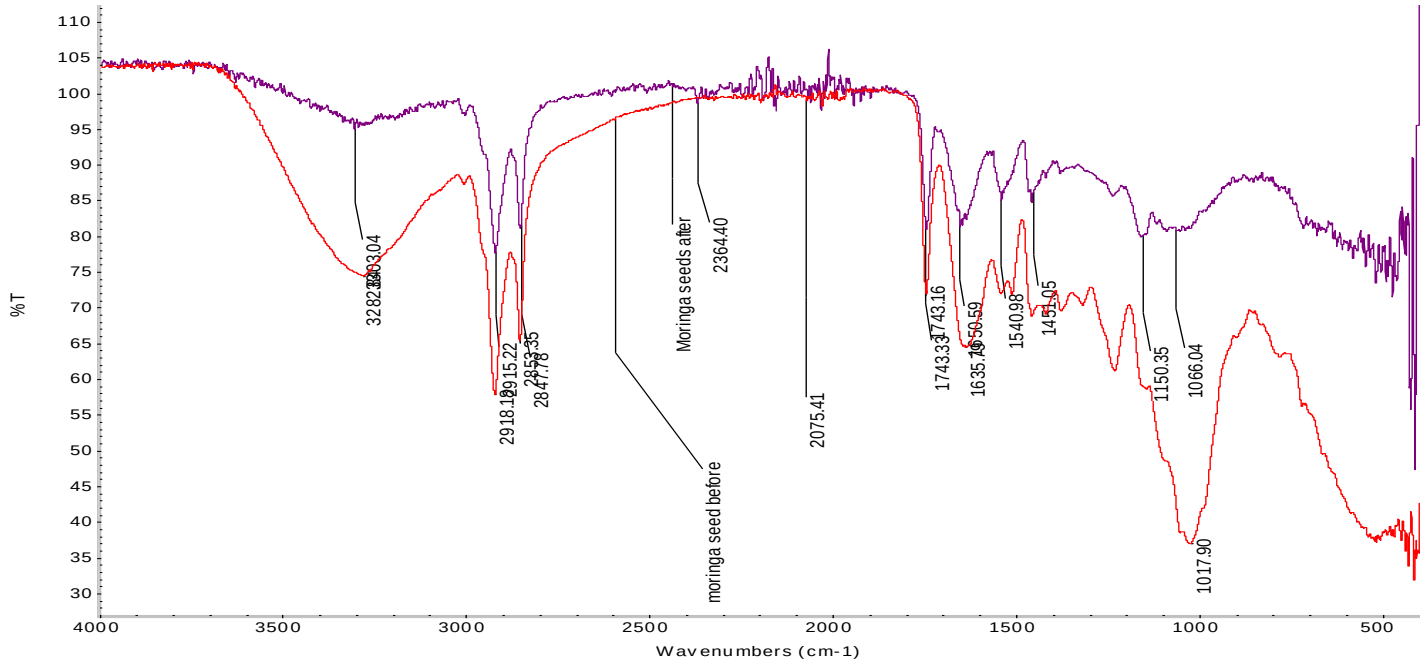

Figure 3. Pre (red) and post (purple) IR spectrum of the *Moringa oleifera* seeds.

shifted because of the C-H stretching. Absorption peak at 1743.33 cm^{-1} lost intensity and shifted to 1730 cm^{-1} due to C=O lead absorption.

Figure 4 shows absorption at 3266.63 cm^{-1} in leaves is due to OH stretching vibration (Kumar and Tamilarasan, 2013; Zhang et al., 2013; Sivaraj et al., 2001). The band at 2914.13 and 2849.06 cm^{-1} is due to C-H stretching. The absorption at 1621.61 cm^{-1} could be C=O (carbonyl) stretch and 1528.39 cm^{-1} is due to C=C stretching (Lee et al., 2007). The adsorption peaks at 1234.67 and 1009.79 cm^{-1} can be assigned to C-O group of an acid and an ester, respectively (Sharma and Uma Gode, 2010).

M. oleifera leaves loaded with Pb^{2+} show the absorption peak at 3266.63 cm^{-1} lost intensity and shifted to 3276.97 cm^{-1} due to stretching of the OH group under the influence of Pb^{2+} adsorption. It shows the absorption peaks at 2914.13 and 2846.24 cm^{-1} lost their intensity and shifted to 2917.08 and 2843.06 cm^{-1}, respectively, because of the C-H stretching. Absorption peak at 1621.81 cm^{-1} lost intensity and shifted to 1623.38 cm^{-1}. The repeated shift due to the C-O stretching suggests that C-O might be a functional group that Pb^{2+} can bind and react with it. The obtained results are in agreement with those of Al-Dujaili et al. (2013), with some differences and this might be as a result of different source of *M. oleifera* leaves.

Effect of initial concentration of Pb^{2+} in aqueous solution

In all the biosobent samples, adsorption capacity increased with increase in initial metal ion concentration. This is due to higher interaction between the metal ion and the adsorption site (Farhan et al., 2012). This trend suggests that increase in adsorbate concentration results in increase in number of available molecules per binding site of the adsorbent, thus bringing about a higher probability of binding of molecules to the adsorbent.

Figure 5 shows that the equilibrium concentrations increased with increase in initial concentration of Pb^{2+}. The amount of Pb^{2+} absorbed by moringa seeds, blended, leaves and pods at equilibrium (Q_e) increased from 0.611 to 2.086 mg/g, 0.5884 to 1.9429 mg/g, 0.5348 to 1.7525 mg/g, and 0.5358 to 1.7755 mg/g, respectively, as the initial concentration was increased from 5 to 20 mg/L. The initial concentration provides an important driving to overcome all mass transfer resistances of the Pb^{2+} between the aqueous and solid phases. Hence, a higher initial concentration of Pb^{2+} ions will enhance the sorption process. The Pb^{2+} removal efficiency decreased from 97.79 to 83.44% for *M. oleifera* seeds, 85.58 to 70.1% for leaves, 85.74 to 71.02% for pods, and 94.15 to 77.72% for the *M. oleifera* blended as the lead concentration was increased from 5 to 20 mg/L. The results show that the moringa seeds have greater sorption capacity, however, blended samples have a comparable sorption efficiency.

Effect of adsorbent dosage

The results show that the effects of adsorbent dosage on adsorption are as shown in Figure 6. Percentage removal

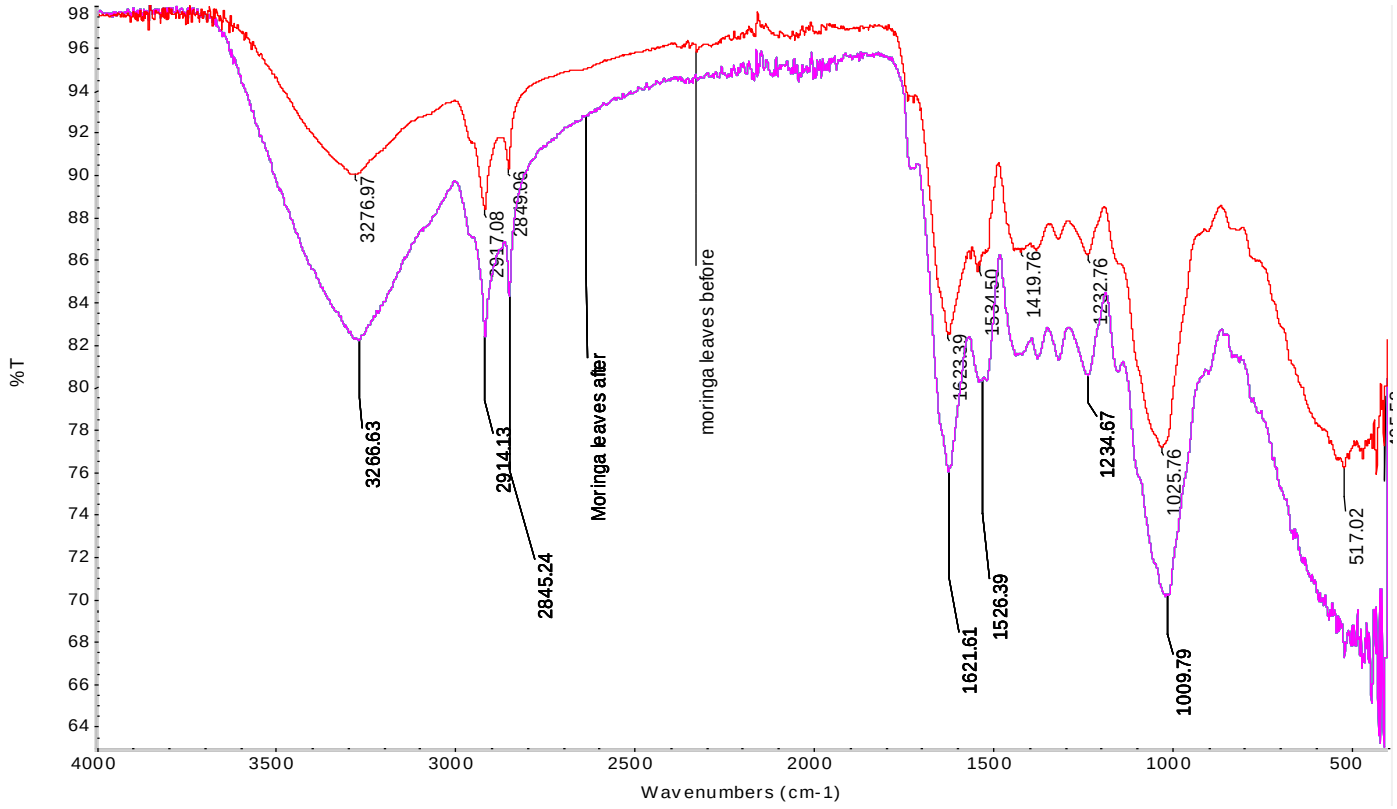

Figure 4. Pre (red) and post (purple) IR spectrum of the *Moringa oleifera* leaves.

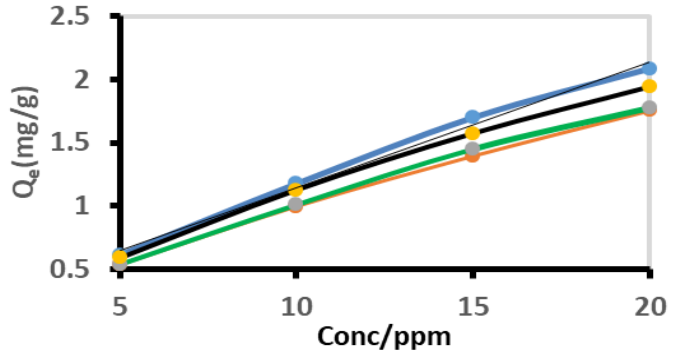

Figure 5. Effect of initial concentration of Pb^{2+} in aqueous solution (seeds-blue, blended-black, leaves-red, and pods-green).

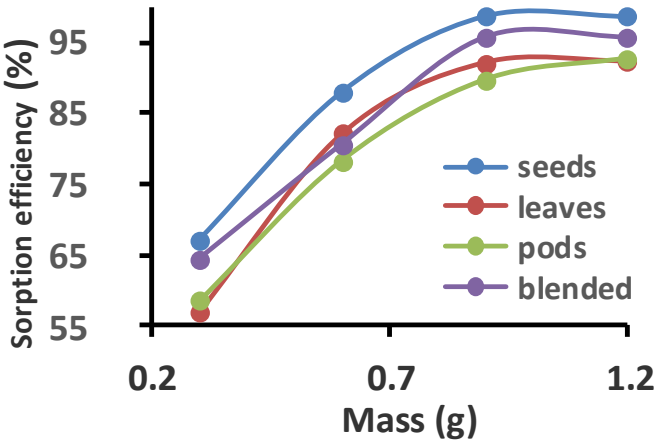

Figure 6. Effect of adsorbent dosage.

increased with increase in dosage up to 0.9 g. The optimum percentage removal for seeds was 98.7%, the blend was 95.85%, leaves was 92.19%, and for the pods was 89.78%. After the optimum dosage, percentage removal becomes constant. However, no significant increment in the biosorption tendency was observed in further increasing the biomass dosage from 0.9 g onward for all the four different biosobent. This can be explained by the fact that at low dosage concentration, there is limited surface area for adsorption, an increase in dosage results in more adsorption sites. After the optimum dosage, active sites for adsorption become excess and the metal ions become limiting and sorption sites remain unoccupied. A direct proportionality relationship between the adsorbent dosage and percent adsorption was also observed when activated carbon prepared from cashew nut shells was used as the sorbent material (Tangjuank et al., 2009).

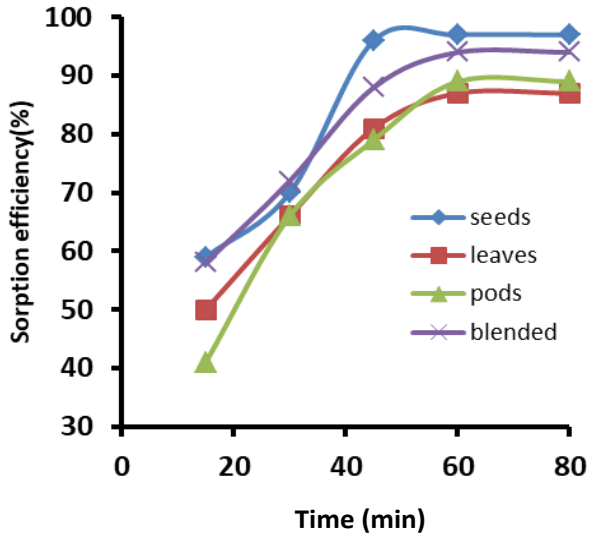

Figure 7. Effect of contact time.

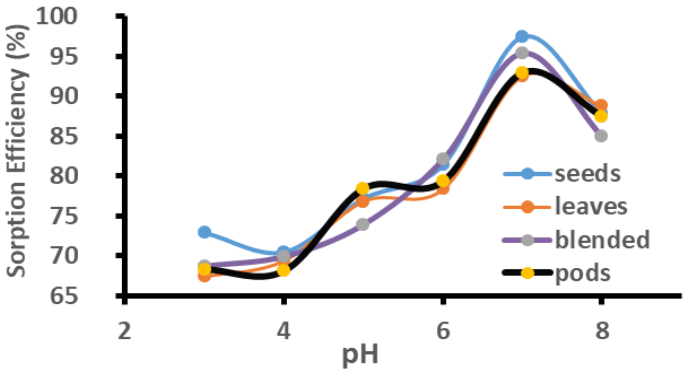

Figure 8. Effect of pH.

Effect of contact time

Percentage removal increased with increase in contact time for all the four biosorbent up to 60 min (Figure 7).

From the adsorption experiment results at 60 min, the sorption efficiency of *M. oleifera* seeds was 96.66%, blend was 93.84%, pods was 88.75%, and leaves was 86.54%. After 60 min, the adsorbed quantities showed nearly no significant change. In the early stages, more number of vacant sites are available for adsorption. As contact time increases, the maximum number of sites that got adsorbed to the metal ions increases which becomes difficult for the metal ions to search for the very few remaining sites, thus rate of adsorption decreases in the later.

Effect of pH

Figure 8 shows the effect of pH on sorption efficiency of the different studied biosorbents.

For all the three biosorbents, the removal efficiency increased with increase in pH. For *M. oleifera* seeds, it increased from 69 (pH 3) to 81% (pH 6) and 97% (pH 7). It increased from 67.4 (pH 3) to 78.83% (pH 6) and 92.49% (pH 7) for *M. oleifera* leaves. It increased from 68.35 (pH 3) to 79.34% (pH 6) and 92.94% (pH 7) for pods. It increased from 68.75 (pH 3) to 82.05% (pH 6) and 95.39% (pH 7) for moringa blend. For all the four biosorptions, there is a general decrease in biosorption from pH 7 to 8. At pH greater than the optimum, there was no significant change in sorption. At low pH, there is competition between H^+ ions and metal ions since the H^+ ions are in high concentration. They tend to protonate the active sites which inhibit the metal ions from binding to the active site. As pH is increased, negatively charged sites increase which result in greater electrostatic attractions (Nharingo et al., 2012). At higher pH, percentage removal is likely to be a result of precipitation of OH^- ions which form complexes with Pb^{2+}. A similar trend was also observed when papaya seeds were used for the removal of zinc (Ong et al., 2011).

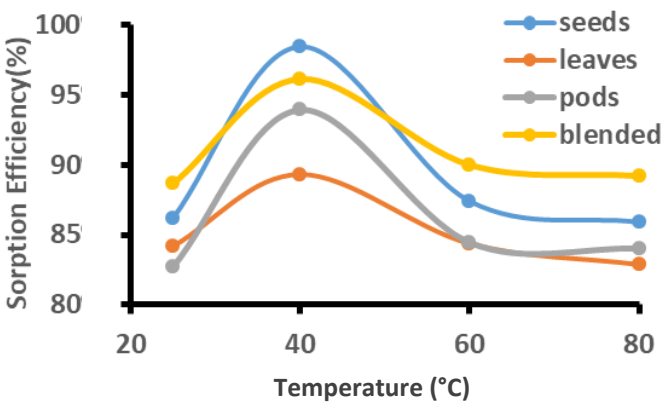

Figure 9. Effect of temperature.

Effect of temperature

The optimum temperature was observed at 40°C with metal removal of 98% for seeds biomass, 96% for moringa blended sample, 94% for pods and 89% for leaves biomass. The data presented in Figure 9 show that the adsorption of metal ions by *M. oleifera* seeds, leaves, pods and the blended biomass increased with increase in temperature which is typical for the biosorption of most metal ions from their solution (McKay et al., 1982).

The magnitude of increase declines as temperature increased from 40 to 80°C. The slight decrease in biosorption with increase in temperature in all samples can be attributed to the attractive forces between biomass surface and the metal ions. The attractive forces

at higher temperatures are likely to be weakened and the sorption decreases. There were significant increases in sorption efficiency of seeds and blended from 25 to 40°C which were 86 to 98% and 89 to 96%, respectively.

Results of the studied biosorbents suggest that Pb^{2+} removal processes from aqueous solutions is biphasic, that is, lower temperature phases (25 to 40°C) and the high temperature sorption process (40 to 80°C). Generally, there is rapid metal sorption process at the lower temperature phase. The sorption of metal ions at higher temperature phases (40 to 80°C) by all the biomasses were very low. At high temperature, the thickness of the boundary layer decreases due to the increased tendency of the metal ion to escape from the biomass surface to the solution phase, which results in a decrease in adsorption as temperature increases (Aksu and Kutsal, 1991).

Sorption isotherms

The purpose of the sorption isotherms is to reveal the specific relation between the equilibrium concentration of adsorbate in the bulk and the adsorbed amount at the surface. The isotherm results of *Moringa oleifera* samples biosorption on lead at a constant temperature of 25°C were analyzed using four important isotherms; the Langmuir, Freundlich, Temkin, and Dubinin–Redushkevich (D–R) isotherm models.

Langmuir isotherm

The linearized Langmuir Equation 2a was used to obtain the isotherm parameters.

$$\frac{C_e}{Q_e} = \frac{C_e}{Q_m} + \frac{1}{Q_m b} \quad (2a)$$

Where, Q_e is the milligrams of accumulated per gram of the biosorbent, C_e is the metal residual concentration in solution, Q_m is the maximum uptake corresponding to the site saturation for a monolayer surface and b is the ratio of adsorption and desorption, that is, it indicates the affinity between adsorbent and the test analyte. A dimensionless Langmuir equilibrium constant (R_L) shown in Equation 3.2b was also used to determine the shape of the isotherms.

$$R_L = \frac{1}{[1 + bC_0]} \quad (2b)$$

Where, R_L values between 0 and 1 show a favourable adsorption process, 0 indicates an irreversible adsorption, 1 indicates a linear adsorption and a value greater than 1 indicates an unfovourable adsorption

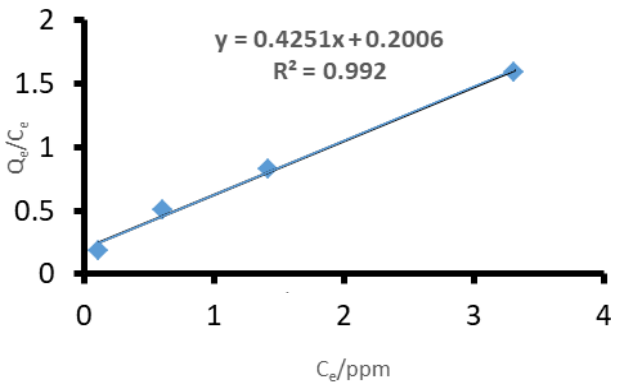

Figure 10. Langmuir plot on biosorption of Pb^{2+} ions by *Moringa oleifera* seeds.

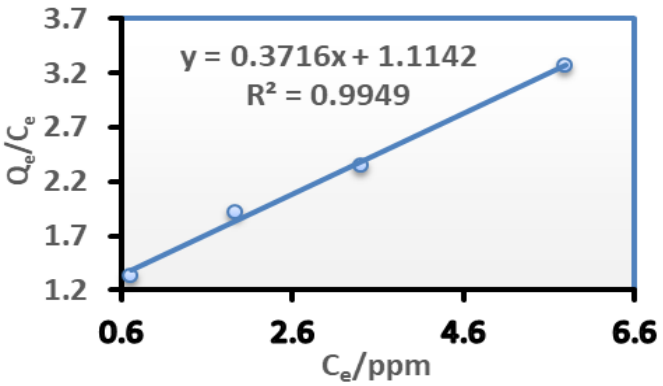

Figure 11. Langmuir plot on biosorption of Pb^{2+} ions by *Moringa oleifera* pods.

process (McKay et al. 1982; Awwad and Salem, 2012). The R_L values were calculated at different concentrations. The Langmuir plots for Pb^{2+} ions sorption using different samples of *M. oleifera* are shown in Figures 10 to 13.

The R_L values are shown in Table 1 and they are within the range of 0 to 1. This shows that the Pb^{2+} biosorption processes were favorable for all *M. oleifera* biosobent (McKay et al., 1982; Awwad and Salem, 2012). The R_L values decreased with an increase in concentration in all *M. oleifera* samples. In *M. oleifera* seeds, the values of R_L were small as compared to all samples at the same concentration and the R_L values were approaching zero with increase in initial concentration which suggest irreversible adsorption with increase in concentration.

The Langmuir constants (Q_m and b) were determined from the slope and intercept of the Langmuir plot and are presented in Table 2.

The correlation coefficients for lead sorption using *M. oleifera* seeds (0.9920), blended (0.9967), pods (0.9949) and leaves (0.9863) are shown in Table 2. They all fit the Langmuir isotherm which indicates good monolayer coverage on the surface of all the biosobents. The

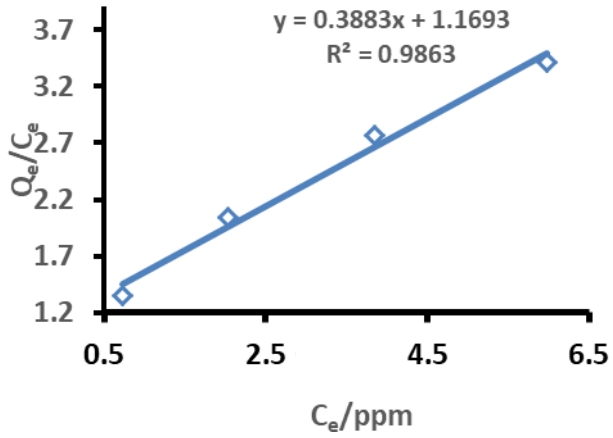

Figure 12. Langmuir plot on biosorption of Pb^{2+} ions by *Moringa oleifera* leaves.

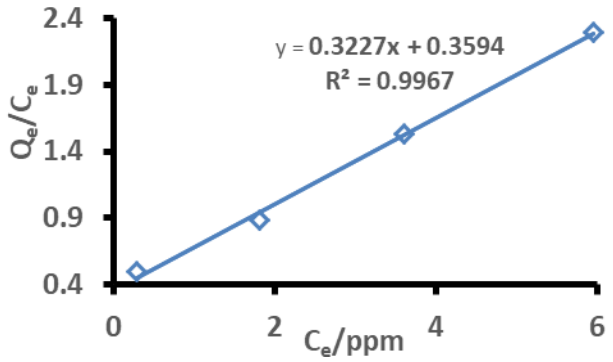

Figure 13. Langmuir plot on biosorption of Pb^{2+} ions by *Moringa oleifera* (blended).

Table 1. R_L Values for seeds, blended, pods, and leaves.

Initial concentration (ppm)	R_L value			
	Seeds	Blended	Pods	Leaves
5	0.0688	0.1822	0.3668	0.3759
10	0.0356	0.1002	0.2246	0.2314
15	0.0240	0.0691	0.1618	0.1672
20	0.0181	0.0527	0.1265	0.1309

Table 2. Langmuir isotherm model parameters.

Parameter	Langmuir isotherm model parameters		
	Q_m (mg/g)	B	R^2
Seeds	2.3524	2.1191	0.9920
Blended	3.0989	0.8979	0.9967
Pods	2.9984	0.3453	0.9949
Leaves	2.5753	0.3321	0.9863

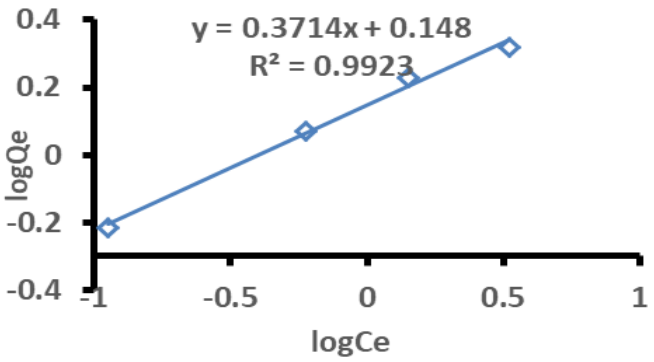

Figure 14. Freundlich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* seeds.

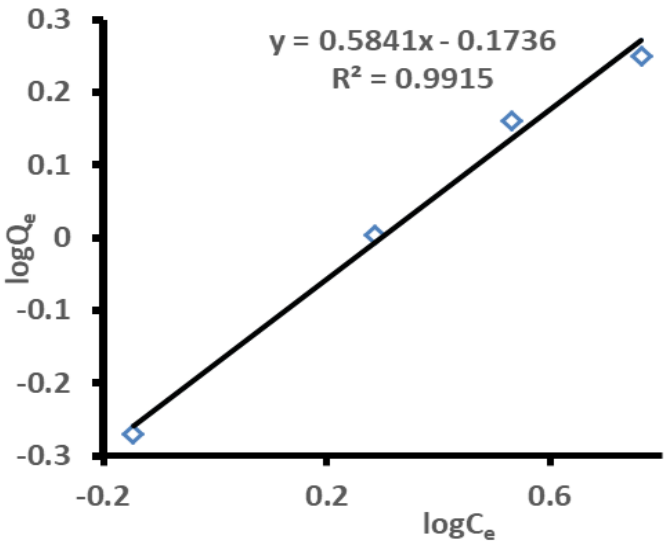

Figure 15. Freundlich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* pods.

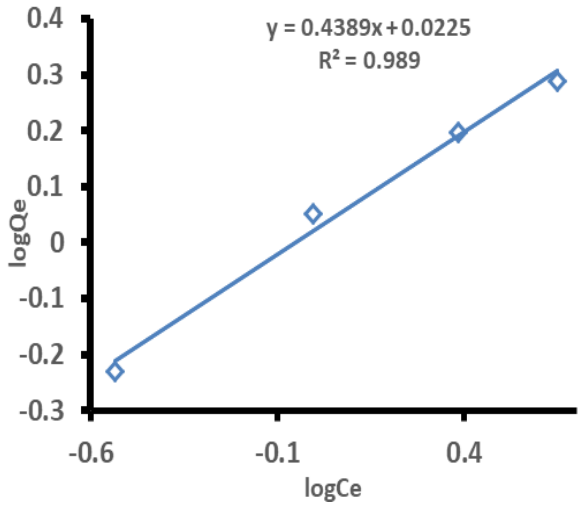

Figure 16. Freundlich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* blended.

saturated monolayer sorption capacity, Q_m is a function of many parameters such as pH and temperature, it provides a good measure for comparing the efficiency of different sorbents in removing a given metal. The sorption capacity, Q_m which is a measure of the maximum adsorption capacity corresponding to complete monolayer coverage showed that the *M. oleifera* samples had a mass capacity for Pb^{2+} in this order: blended (3.0989 m/g) > pods (2.9984 mg/g) > leaves (2,5753 mg/g > seeds (2.3524 mg/g). These values are higher than those obtained in other studies for Pb^{2+} adsorption (Aziz et al., 2016). The order of moringa maximum sorption capacity proved that the blended sample has better sorption capacity at 25°C. The b-values for seeds, blended, pods and leaves were 2.1191, 0.8979, 0.3453, and 0.3321, respectively. The positive values of b show that the biosorption of Pb^{2+} by *M. oleifera* samples was endothermic (Deng et al., 2008).

Freundlich isotherm

The linear form of the Freundlich isotherm Equation 3 was used to estimate the adsorption intensity of the sorbent towards the adsorbate. It assumes that the adsorption process takes place on a heterogeneous surface of adsorption capacity.

$$\log Q_e = \log K_F + \frac{1}{n}\log C_e \quad (3)$$

Where, Q_e is the metal uptake at equilibrium concentration (ppm), C_e is the equilibrium metal ion concentration (ppm), K_F is the Freudlich's constant of adsorption capacity and n is the constant of adsorption. K_F was estimated from the y-intercept and n was calculated from the slope. Plots for the determination of Freundlich isotherm parameters are presented in Figures 14 to 17.

The Freundlich isotherm parameters (R^2, K_F, and n) extracted from Figures 14 to 17 are shown in Table 3. Low values of K_F indicate minimal adsorption (Ashraf et al., 2012). According to K_F values, the order of ascending adsorption was seeds (2.6925)> blended (2.2784)> leaves (1.78)> pods (1.7120). The n-values should be in the range 1<n<10 to satisfy the heterogeneity condition (Farhan et al., 2012). This also implies a beneficial adsorption process. The n-values for all the moringa samples on Pb^{2+} sorption fall in this range, therefore, the surfaces are heterogeneous. The n-values are greater than 1 which shows that the adsorptions were mainly physical processes for all the samples (Farhan et al., 2012). According to n-values in Table 3, the order of heterogeneity was increasing in the order: seeds (2.6925)>blended (2.2784)>leaves (1, 7806)>pods (1.712).

Analysis of the *n*-values in Table 3 also shows that

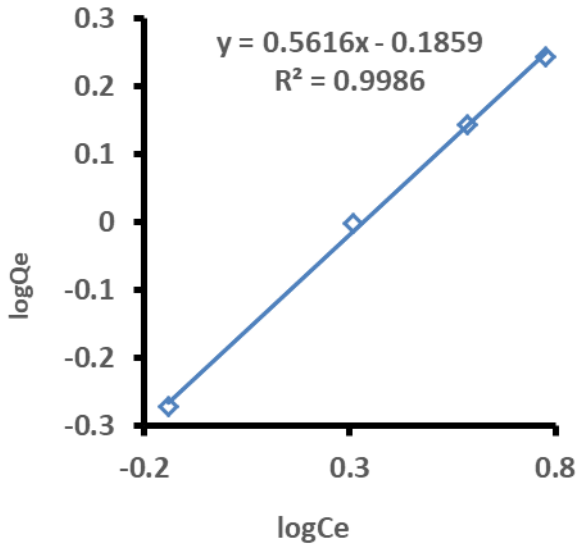

Figure 17. Freundlich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* leaves.

Table 3. Freundlich isotherm parameters.

Parameter	Freundlich isotherm		
	n values	K_f values	R^2
Seeds	2.6925	1.4060	0.9923
Blended	2.2784	1.0532	0.9890
Pods	1.7120	0.6705	0.9915
Leaves	1.7806	0.6705	0.9890

adsorption of Pb^{2+} into moringa seeds, blended, pods and leaves much favored physical adsorption process on Pb. Adsorption capacity on seeds and blended sample was likely to promote new chemical bonds at the biomass's surface which might lead to the enhancement of the biomass adsorption capacity. This reasoning could explain the cause of the increase of removal efficiency with the increase of initial Pb^{2+} concentration and the higher values of K_F (adsorption capacity) on moringa seeds and blended sample compared to moringa leaves and pods. However, physical adsorption was shown to be favorable for adsorption of Pb^{2+} by all the biomas samples. In some studies, Pb^{2+} ions adsorption has been reported to favour chemisorption (Aziz et al., 2016). This can be attributed to different sources of the *M. oleifera* plant in which different geographical location and climatic conditions can affect the chemical composition of the biosorbent.

Dubinin-Radushkevich (D-R) isotherm

The D-R isotherm model was applied to the data in order to deduce the heterogeneity of the surface energies of adsorption and the characteristic porosity of the adsorbent. The linear form of the D-R isotherm is given in Equation 4a:

$$\ln q_e = \ln q_D - B_D\left[RT\ln(1+\frac{1}{C_e})\right]^2 \quad (4a)$$

Where, q_D is the amount of lead adsorbed per unit dosage of *M. oleifera* samples (mol/g), q_e is the theoretical monolayer sorption capacity (mol/g), B_D is the constant of the sorption energy (mol^2/J^2), which is related to the average energy of sorption per mole of the adsorbate as it is transferred to the surface of the solid from infinite distance in the solution (Dubinin et al., 1947), T is the solution temperature (K) and R is the gas constant. The value of mean sorption energy (apparent energy of adsorption), E (kJ/mol), was calculated from D-R parameter according the relationship represented in Equation 4b:

$$E = \frac{1}{\sqrt{2B_D}} \quad (4b)$$

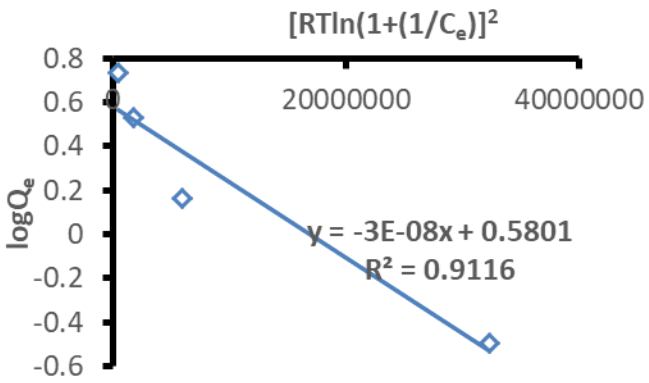

Figure 18. Dubinin-Radushkevich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* seeds.

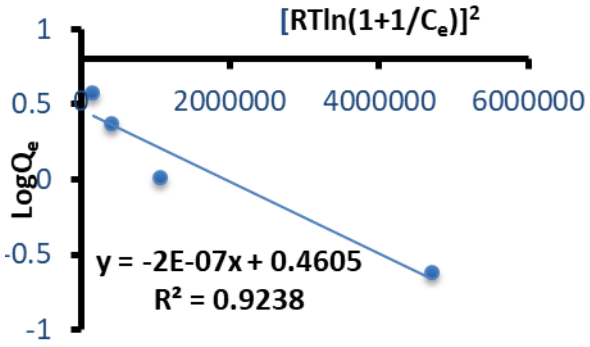

Figure 19. Dubinin-Radushkevich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* pods.

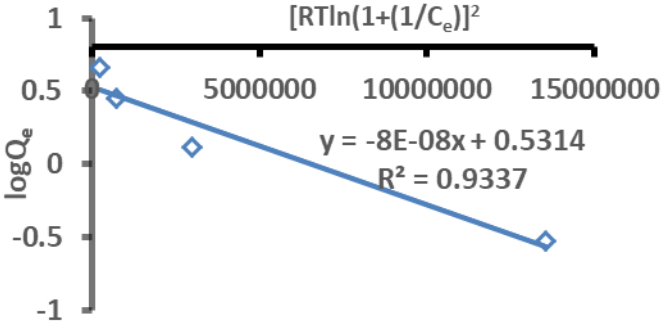

Figure 20. Dubinin-Radushkevich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* blended.

The value of mean sorption energy gives information about chemical and physical sorption. The *E* value ranges from 1 to 8 kJ/mol for physical sorption and from 8 to 16 kJ/mol for chemical sorption (Zheng et al., 2008). The D-R plots ($\ln Q_e$ against $[RT\ln(1+1/C_e)]^2$) for the determination of D-R isotherm parameters are presented in Figures 18 to 21.

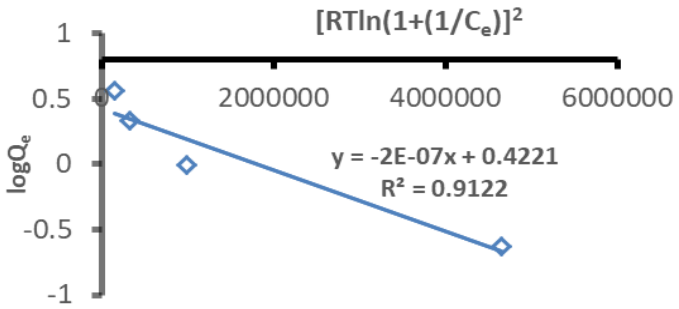

Figure 21. Dubinin-Radushkevich plot on biosorption of Pb^{2+} ions by *Moringa oleifera* leaves.

The D-R isotherm parameters are shown in Table 4. The maximum sorption capacity of lead ions on moringa samples (q_D) was in the order: moringa seeds (1.786 mol/g) > moringa blended (1.701 mol/g) > moringa pods (1.585 mol/g) > moringa leaves (1.525 mol/g) at 25°C. The E-value (4.082, 2.5, 1.581, and 1.525 KJ/mol) for moringa seeds, moringa blended, moringa pods, and moringa leaves, respectively. These were all found in the range of 1 to 8 kJ/mol, indicating that the type of sorption of Pb^{2+} on all moringa samples is essentially physical process.

Temkin isotherm

The Temkin model assumes that the heat adsorption of all molecules decreases linearly with coverage due to adsorbate-adsorbent interactions. Its linear form is expressed as shown in Equation 5.

$$Q_e = B\ln A + B\ln C_e \quad (5)$$

Where, $B = \frac{RT}{b_T}$ in J/mol corresponding to the heat of adsorption, b_T is the Temkin isotherm constant, A is the equilibrium binding constant for the maximum binding energy.

Plots for the determination of Temkin isotherm parameters are as shown in Figures 22 to 24. The constants *A* and b_T were calculated from the intercept and slope of the Temkin plot and are listed in Table 5.

For analysis of Temkin isotherm parameters in Table 6, equilibrium binding constant were in the order: seeds > blended > pods > leaves. The results reflected that the seeds have a greater binding constant and the blended sample yielding almost the same binding constant. The constant b_T values were 5638.53 (seeds), 5025.50 (blend), 4144.48 (pods) and 4383 J/mol (leaves). This is an indication of the heat of sorption indicating a physical adsorption.

Table 4. D-R isotherm parameters.

Parameter	D-R parameters			
	q_D (mol/g)	B_D (mol^2/J^2)	E (KJ/mol)	R^2
Seeds	1.786	3×10^{-8}	4.082	0.9116
Blended	1.701	8×10^{-8}	2.500	0.9337
Pods	1.585	2×10^{-7}	1.581	0.9238
Leaves	1.525	2×10^{-7}	1.525	0.9122

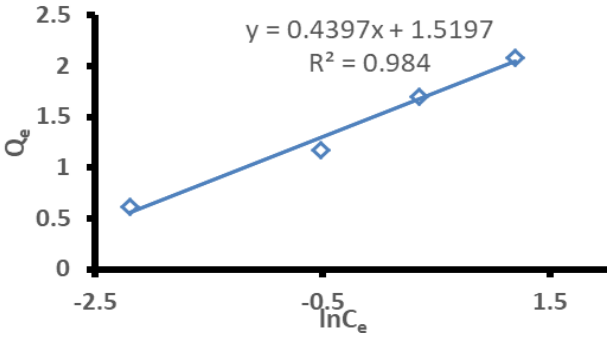

Figure 22. The linear plot of Temkin isotherm of *Moringa oleifera* seeds.

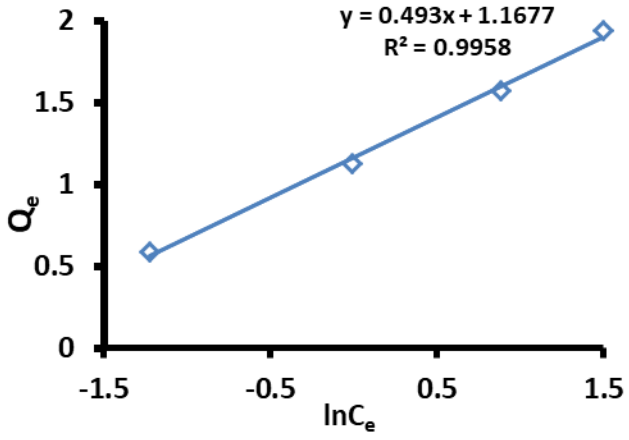

Figure 23. Temkin isotherm of *Moringa oleifera* blended.

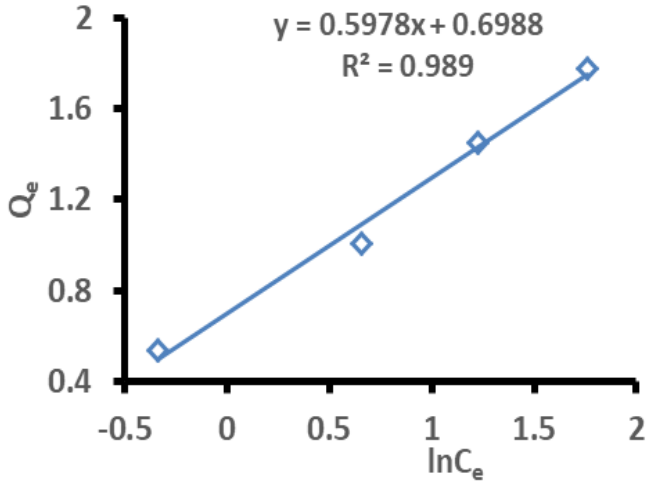

Figure 24. Temkin isotherm of *Moringa oleifera* pods.

Kinetic study of Pb^{2+} adsorption

The pseudo-first order and pseudo-second order kinetic models were used to investigate mechanisms of Pb^{2+} ions onto different parts of *M. oleifera* plant. Lagergren proposed a pseudo first order kinetic model represented in Equation 6.

$$\log(q_e - q_t) = \log q_e - \frac{k_{ad}}{2.303}t \quad (6)$$

Where, q_t and q_e are adsorption capacity at time t and at equilibrium, respectively, and k_{ad} is the pseudo-first order rate constant of adsorption. The linear plot of log(qe-qt) vs. t (Figure 26) was used to estimate the rate constant and correlation coefficients for Pb^{2+} adsorption. The pseudo-second order reaction parameters were estimated using the Equation 7.

$$\frac{t}{q_t} = \frac{1}{h} + \frac{1}{q_e}t \quad (7)$$

Where, $h = kq_e^2$ (mg g^{-1} min^{-1} and k = rate constant for pseudo-second order adsorption mechanism. The linear plot of t/qt vs. t (Figure 27) was used to estimate the pseudo-second order rate constants and correlation coefficients for Pb^{2+} adsorption. These parameters are summarised in Table 6. The highest rate constant for the pseudo-first order kinetic model was obtained from the seeds. The blended sample gave the highest rate constant for the pseudo-second order kinetic model. The correlation coefficients are generally low for both kinetic models although the pseudo-second order kinetic model gave relatively higher values. Such low values can be attributed to more physical adsorption interactions than chemisorption.

Table 5. Temkin isotherm parameters.

Parameter	A(*(L/g)*	b_T(*(J/mol)*	R^2
Seeds	31.697	5638.53	0.984
Blended	10.68	5025.50	0.9958
Pods	3.319	4144.48	0.989
Leaves	3.284	4383.53	0.9822

Table 6. Estimated rate constants and correlation coefficients.

Parameter	First-order reaction parameters		Second-order reaction parameters	
	k_{ad}	R^2	k	R^2
Seeds	0.122	0.8246	5.0×10^{-4}	0.9759
Blended	0.060	0.9404	5.9×10^{-4}	0.9930
Pods	0.052	0.9988	3.0×10^{-4}	0.9833
Leaves	0.061	0.9570	5.5×10^{-4}	0.9922

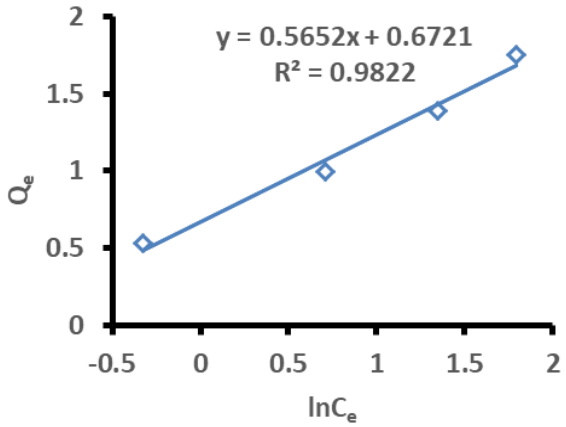

Figure 25. Temkin isotherm of *Moringa oleifera* leaves.

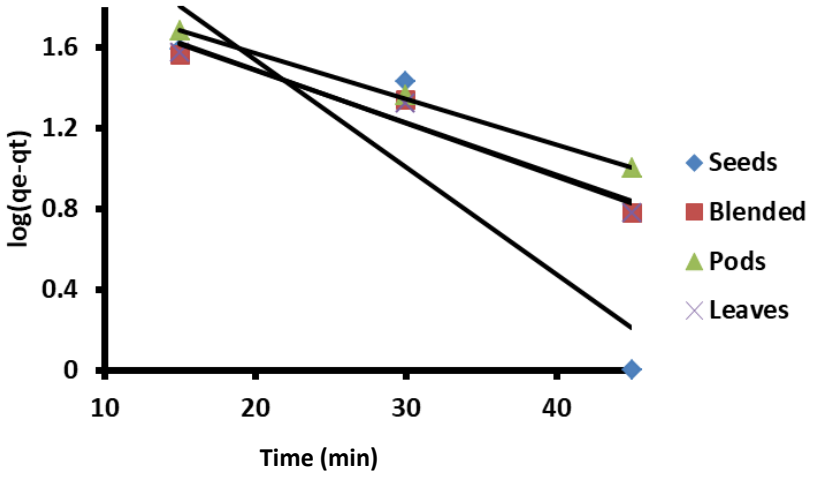

Figure 26. Pseudo-first order reaction for Pb^{2+} adsorption onto different *Moringa oleifera* plant parts.

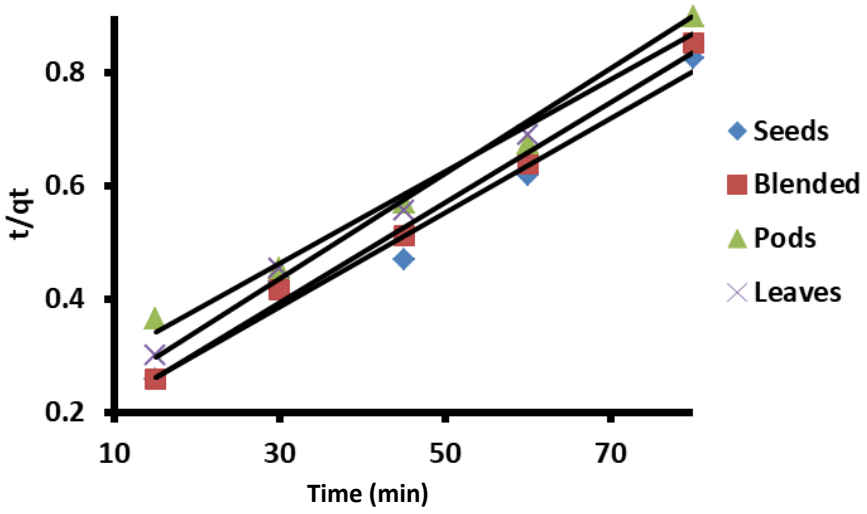

Figure 27. Pseudo-second order reaction for Pb^{2+} adsorption onto different *Moringa oleifera* plant parts.

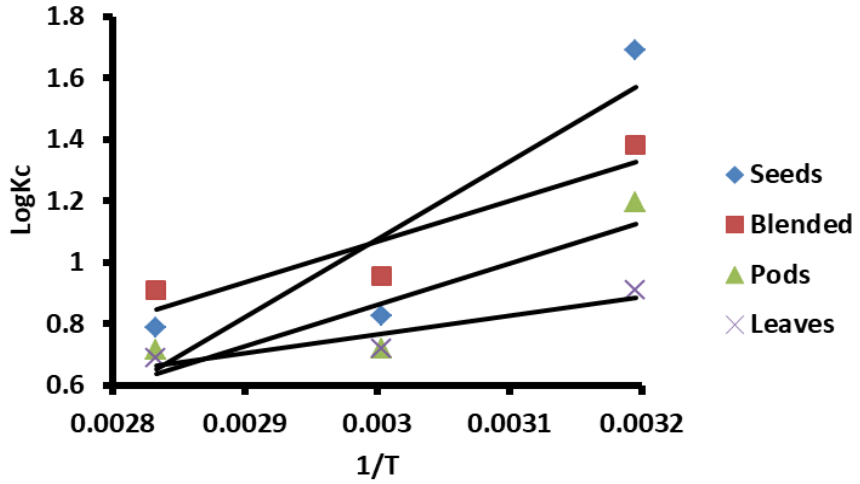

Figure 28. Thermodynamic plots for Pb^{2+} adsorption onto different *Moringa oleifera* plant parts.

Estimation of Gibbs free energy (ΔG°), enthalpy (ΔH°), and entropy (ΔS°) for Pb^{2+} adsorption

These thermodynamic parameters were evaluated using Equation 8a to c.

$$K_C = \frac{C_{e_{SP}}}{C_e} \quad (8a)$$

$$\Delta G° = -RT \ln K_C \quad (8b)$$

$$\log K_C \frac{\Delta S°}{2.303R} - \frac{\Delta H°}{2.303RT} \quad (8c)$$

Where, Kc = equilibrium rate constant, Cesp = solid phase concentration at equilibrium (mg/L), Ce = equilibrium concentration in solution (mg/L), and ΔG°, ΔH°, ΔS° are changes in Gibbs free energy (kJ/mol), enthalpy (kJ/mol), and entropy (J/mol/K). A linear plot of logKC vs. 1/T (Figure 28) was used to estimate ΔH0 and ΔS0 for Pb^{2+} adsorption on *M. oleifera* plant parts. The thermodynamic parameters are summarised in Table 7.

ΔG° values for all adsorbents were negative (Table 7), indicating that the adsorption process is feasible and spontaneous. The seeds have shown an enhanced feasibility of Pb^{2+} adsorption at 40°C, however this decreased at higher temperatures. The blended sample also has comparable adsorption feasibility. The ΔG° values for all samples were less than -20 kJ/mol, indicating physical adsorption as the main interaction

Table 7. Estimated thermodynamic parameters.

Parameter	ΔH° (kJ/mol)	ΔS° (J/mol/K)	ΔG° (kJ/mol)			
			25°C	40°C	60°C	80°C
Seeds	-48.5	-125.0	-4.48	-10.12	-5.26	-5.31
Blended	-25.4	-55.6	-5.78	8.28	-6.09	-6.13
Pods	-25.8	-61.0	-3.94	-7.16	-4.60	-4.84
Leaves	-11.8	-20.6	-4.11	-5.44	-4.60	-4.58

between Pb^{2+} and all the adsorbents. However, in another study by Adebisi et al. (2016), ΔG° values for Pb^{2+} adsorption using activated carbon from palm oil mint effluent were positive hence less spontaneous. This shows that spontaneity of lead adsorption depends so much on type of biomass. The ΔH° values in this study were all negative, indicating the exothermicity of the adsorption process. The ΔS^0 values revealed that the randomness of the adsorption process was more pronounced with the seeds (-125.0 kJ/mol).

Conclusion

Moringa leaves, pods, seeds were all found to be potential biosorbents for the removal of Pb^{2+} ions from aqueous solution. The percentage of moringa biomass recovery for different sample were 12.9% for leaves, 5.45% for pods, 23.5% for seeds, and ash was 22.3%. Characterisation of the moringa samples by the FTIR showed the presence of OH, carboxylic acid, ester, ketone or aldehyde C=O group, C-O group, methyl functional group and C=C group. These were involved in Pb^{2+} biosorption on all moringa biomas samples. The results indicated the optimum pH of 7 for Pb^{2+} ions biosorption with the sorption capacities of 97% for seeds, 92.94% for leaves, 95.39% moringa blended and 92.94% for moringa pods. The sorption data fitted into Temkin, Dubunin-Radushkevich, Langmuir and Freundlich isotherms. The Langmuir adsorption model was found to be having the best regression value for all the moringa samples and hence the overall best fit. Thermodynamic and kinetic data indicated good adsorption properties for the seed and blended samples. It could be settled that the seed has great potential to remove Pb^{2+} ions from waste water, however, the blended sample proved to be the best biosorbent for removal of Pb^{2+} ions from its aqueous solution with respect to its abundance and cost considerations. These good sorption properties reported here together with known medicinal properties of *M. oleifera* shows great potential for application in cartridges/columns to be used in cleaning of domestic waters.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Adebayo PD, Adebayo AA, Adediji JF, Adebayo OT (2012). Isotherm, kinetic and thermodynamic study of Lead (II) biosorption by *Streblus asper*. Pac. J. Sci. Technol. 13(2): 283-291.

Aksu Z, Kutsal TAA (1991). Bioseparation process for removing Pb(II) ions from wastewater by using *C. vulgaris*. J. Chem. Technol. Biotechnol. (52)(1):108-118.

Al-Dujaili AH, Awwad AM, Salem NM (2012). Biosorption of cadmium (II) onto loquat leaves (*Eriobotrya japonica*) and their ash from aqueous solution, equilibrium, kinetics, and thermodynamic studies. Int. J. Ind. Chem. 3:1-7.

Ashraf MA, Maah M J, Yusoff L (2012). Removal of lead from synthetic solutions by protonated Teleosts. Int. J. Environ. Sci. Technol. (9)1:34-353.

Awwad AM, Salem NM (2012). Biosorption of copper(II) and lead(II) ions from aqueous solutions by modified loquat (*Eriobotrya japonica*) leaves. J. Chem. Eng. Mat. Sci. 3(1):7-17.

Aziz NAA, Jayasuriya N, Fan L (2016). Adsorption study on *Moringa oleifera* seeds and Musa cavendish as natural water purification agents for removal of Lead, Nickel and Cadmium from drinking water. Mat. Sci. Eng. 136:1-9.

Babarinde NAA, Bablola JO, Adegoke J, Muraizu U, Ongumbawo T, Ogunjinrin E (2012). Kinetic, equilibrium and thermodynamic solution using banana (*Musa acuminata*) leaf. Int. J. Phys. Sci. 7(9):1376-1385.

Das N, Vimala R, Karthika P (2008). Biosorption of heavy metals. Indian J. Biotechnol. 7(1):159-169

Deng L, Su Y, Su H, Wang X, Zhu X (2008). Biosorption of lead and copper by non-living green algae *Cladofera fascicalaris*: Equilibrium, kinetics and environmental effects. Adsorption 12: 267-277.

Dubinin MM, Zaverina ED, Radushkevich LV (1947). Sorption and Structure of active carbon I. Adsorption of organic vapours, J. Phys. Chem. 21:1351-1352..

El-Sayed, GO, Dessouki HA (2011). Removal of Zn(II), Cd(II) AND Mn(II) from aqueous solution by adsorption on maize stalks. Malays. J. Anal. Sci. 15(1):8-21.

Farhan AM, Saleem NM, Ahamad AL, Awwad AM (2012). Kinetic equilibrium thermodynamics studies of biosorption of heavy metals by ceratonia siliqua bark. Am. J. Chem. (2)6: 335-342.

Igwe JC, Abia AA (2006). A Bioseparation for removing heavy metals from waste water using biosorbents. Afr. J. Biotechnol. 5:12.

Kakalanga SJ, Jabulani XB, Olutoyi OB, Utieyin OO (2012). Screening of Agricultural Waste for Ni (II) adsorption: Kinetics, equilibrium and thermodynamic studies. Int. J. Phys. Sci. 7(17):2525-2538.

Kaya C, Ashraf M, Sonmez O, Aydemir S, Tuna AL, Cillu MA (2009). The influence of Arbuschular mycorrhizal colonisation on key growth parameters and fruit yield of pepper plants grown at high salinity. J. Sci. Hortic. 121(1):1-6.

Khan MA, Khan RAR, Mohammed A (2008). Heavy metal pollution and its control through non convectional adsorbents (1998-2007): A review. J. Int. Environ. App. Sci. 3(2):101-114.

Kumar M, Tamilarasan R (2013). Removal of Victoria Blue using *Prosopis juliflora* bark carbon: Kinetics and thermodynamic modelling studies. J. Mat. Environ. Sci. 5 (2):510-519.

Lee CK, Ong ST, Zainal Z (2007). Removal of organic dyes by acid treat spent bleaching earth. Bioresour. Technol. 98:2792-2799.

Mahmood T, Salman AM, Syed TH (2010). Biosorption and recovery of

heavy metals from aqueous solutions by water hyacinth ash. Bioresour. J.: 5(2):1244-1256.
McKay G, Blair HS, Findon A (1982). Equilibrium studies for the sorption of metal ions onto chitosan. Ind. J. Chem. Section A. 28:356-360.
Memon JR, Memon SQ, Bhanger MI, Khuhawar Y (2008). Banana Peel: A green and economical sorbent for Cr(III) removal. Pak. J. Anal. Environ. Chem. 9(1): 20-25.
Nharingo T, Muzondo NM, Madungwe E, Chigondo F, Guyo U, Nyamuda B (2012). Isotherm study of the biosorption of Cu (II) from aqueous solution by *Vigna* Subterranean (L.) Verdc Hull. Int. J. Sci Technol. Res. 1(1):25-33.
Oliveira JDAN, Candido Siqueira LM, de Sousa Neto JA, Alves VN, Coelho NMM (2017). Preconcentration system for determination of lead in chicken feed using *Moringa oleifera* as a biosorbent. Microchem. J. 133: 327-332.
Ong ST, Yip S, Keng P, Lee SL, Hung YT (2011). Papaya (Carica papaya) seed as a low cost biosorbent for zinc removal. J. Agric. Res. 7(5): 811.
Park D, Yun YS, Park MJ (2010). The past, present and future trends of biosorption. Biotechnol. Bioprocess Eng. 15(1):86-102.
Qaiser S, Saleemi AR, Muhammad U (2009). Biosorption of lead (II) and chromium (VI) on groundnut hull: Equilibrium kinetics and thermodynamic study. Elec. J. Biotechnol. 12(4):1-17.
Safa Y, Bhatti HN (2011). Kinetic and thermodynamic modeling for removal of Direct Red-31 and Orange-26 dyes from aqueous solution by rice husks. Desalination 272:313-322.
Sharma YC, Uma Gode FJ (2010). Batch adsorption studies for the removal of Cu(II) Ions by Zeolite NaX from aqueous stream. Chem. Eng. Data. 55:3991.
Sivaraj R, Namasivayam C, Kadirvelu K **(**2001). Orange peel as an adsorbent in the removal of acid violet 17 (acid dye) from aqueous solutions. Waste Manag. J:105-11.
Tangjuank S, Insuk N, Udeye V, Tontrakoon J (2009). Chromium (III) sorption from aqueous solutions using activated carbon prepared from cashew nut shells. Int. J. Phys. Sci. 4:412-417.
Volesky B (2003). Biosorption: Application aspects and process modelling tools. Hydrometallurgy J. 7(1-2):179-185.
World Health Organization (1984). Guidelines for drinking water quality vol. 1-2, World Health Organization, Geneva, Switzerland.
Yakan E, Gavusoglu K, Maras M, Biyikoglu M (2008). Biosorption of Pb(II) and Cu(II) metal ions on *Cladophora glomerata* (L)kutz.(Chlorophytal) Algae: Effect of algal surface modification. Acta Chim. Slov. 55:82-87.
Yang C, Wang J, Lei M, Xei G, Zeng G, La S (2009). Biosorption of zinc from aqueous solution by dried activated slugde. J. Environ. Sci. 22(5):675-680.
Zhang Z, O'Hara IM, Kent GA, Doherty WOS (2013). Comparative study on adsorption of two cationic dyes by milled sugarcane bagasse. Ind. Crops Prod. J. 4: 41-49.
Zheng H, Wang Y, Zheng Y, Zhang H, Liang S, Long M (2008). Equilibrium, kinetic and thermodynamic studies on the sorption of 4-hydroxyphenol on Cr-bentonite. Chem. Eng. J. 143:117-123.

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Variability of seed oil content and fatty acid composition in Shantung maple (*Acer truncatum* Bunge) germplasm for optimal biodiesel production

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Shantung maple seed oil methyl esters have emerged as the potential feedstock for producing biodiesel. The goal of this work was to assess variations in seed oil content and fatty acid compositions for optimal biodiesel production among 138 Shantung maple accessions native to 14 regions of China. Dramatic differences in seed oil content were observed among trees grown in the various regions tested; seeds of trees grown in Daiqintala, Inner Mongolia (DQTL) and Yongshou, Shaanxi (YS) exhibited the highest oil content (32.47 and 32.09%, respectively). Among the 138 germplasm accessions, seed oil content ranged from 17.81 to 36.56%, with a mean value of 28.57%. Of a total of 15 fatty acid components detected overall, oleic acid and linoleic acid comprised the highest proportions of fatty acids (20 to 34.31% and 27.08 to 36.71%, respectively). Correlation analysis revealed the highest positive correlation between oleic acid and cis-11-eicosenoic acid (0.698) and the highest negative correlation between oleic acid and linoleic acid (-0.766). Ranges of saponification number (180.26 to 182.86), iodine value (101.84 to 113.70 g I_2/100 g), cetane number (50.77 to 53.53), density (873.03 to 880.08 kg/m^3) and kinematic viscosity (4.92 to 5.28 mm^2/s) confirmed that Shantung maple methyl esters are suitable for biodiesel production, and correlation analysis showed that the accession with high monounsaturated fatty acid content was suitable as optimal germplasm resources for biodiesel production. DQTL, YS and Taian, Shandong (TA) regions was considered the best plantation, and DQTL-1, DQTL-6, DQTL-8, YS-6, and TA-10 germplasm accessions generated oil with optimal properties for biodiesel production. These results could guide future development of Shantung maple seed oil for improved biodiesel production.

Key words: *Acer truncatum* Bunge, biodiesel properties, fatty acid composition, oil content, variation.

INTRODUCTION

Shantung maple (*Acer truncatum* Bunge), a deciduous tree of northern China belonging to the *Aceraceae* family, is a popular landscape plant due to its brilliant autumn leaf color and a kind of afforestation tree species (Li et al., 2015; Zhao et al., 2007). It is also called the ingot maple because of the samaras like the gold ingot of Chinese. Its sturdy wood texture promotes its use for timber, while its high seed yield and high protein,

chlorogenic acid, tannin and flavonoids content (Ma et al., 2005; Honma et al., 2010) make it an attractive source of protein, medicinal and chemical raw materials. The main chemical ingredients of Shantung maple seeds are lipids (47.88%), protein (27.15%), and minor amounts of sucrose (6.10%) and cellulose (3.68%) (Wang, 2013). Moreover its seed oil has long been used as a food source in northeastern China, but has not yet been used on a large scale in the market. Previous studies (Hu et al., 2017) demonstrated that *A. truncatum* seed oil (ATO) contains 92% unsaturated fatty acids, including 6.22% nervonic acid. This is noteworthy because nervonic acid is an uncommon fatty acid used to treat various neurological disorders, including schizophrenia (Akoh and Moussata 2001) and psychosis (Evans et al., 2003). It has a high vitamin E content of 125 mg/100 g (Wang and Wang, 2011). Currently, the Shantung maple is receiving much attention from Chinese researchers because of its huge potential as an energy source and other uses. Its fruit is currently used to generate renewable biomass energy, yielding 30 kg of fruit per tree after 20 years (Wang, 2013). Meanwhile, ATO has been approved as a new food resource by the Chinese Ministry of Health in 2011. Due to its numerous emerging uses, the farmed Shantung maple cultivation area now encompasses 4×10^4 ha and is rapidly expanding, with continued rapid growth anticipated. Currently, it has been included in the national development plan to use Shantung maple seeds to develop new resources for food and biomass energy.

As mankind's current rapid economic developmental pace, energy shortages and environmental pollution challenges have become more and more apparent worldwide. Regarding energy production, seed oil fatty acid methyl esters (FAMEs) have already served as a suitable diesel engine fuel source (Harrington, 1986; Azam et al., 2005; Karmakar et al., 2010). Woody oil tree species do not occupy arable land and do not compete with the food sector, hence their name biomass energy tree species. Meanwhile, green diesel fuels produced from woody plants, such as *Prunus scoparia* (Sorkheh et al., 2016), *Jatropha curcas* (Sinha et al., 2015), *Pongamia pinnata* (Mukta et al., 2009), *Sapindus saponaria* (Lovato et al., 2014) and *Xanthoceras sorbifolium* (Yu et al., 2017), are now receiving increasing attention as environmentally friendly, non-toxic and biodegradable petroleum diesel fuel substitutes. Concurrently, higher oil content has been shown to improve biodiesel yield and economic feasibility. This knowledge has spurred initial evaluation of new higher oil-containing woody tree seeds, including ATO, as biodiesel sources. In fact, ATO has been found as a suitable source of fatty acids for potential biodiesel used by extensive studies in China, and the oil conversion rate can reach more than 99% (Wei et al., 2008; Liu and Wang, 2009). Now, some potential biofuel crops have been launched in the variation of oil content and fatty acid composition to screen for optimal germplasm, such as *J. curcas* (Kaushik and Bhardwaj, 2013), *P. pinnata* (Mukta et al., 2009), *X. sorbifolium* (Yu et al., 2017) and others. However, there is almost no study on the variation of biodiesel characteristics of Shantung maple seed oil. Most researchers were concerned with the ecological characteristics (Song et al., 2016), oil extraction method (Hu et al., 2017), and the effect of reaction conditions and catalyst types on biodiesel properties (Wei et al., 2008; Liu and Wang, 2009). Therefore, it is vitally important to screen the germplasm based on its oil content and fatty acid composition to obtain high-quality biodiesel for the future (Sinha et al., 2015; Yu et al., 2017).

The aim of this work was to evaluate the biodiesel properties of *A. truncatum* seed oil fatty acid methyl esters by analyzing the variability of oil content and fatty acid composition of various germplasm accessions grown in various regions of China and also screen the optimal germplasm for breeding.

MATERIALS AND METHODS

Plant material

One hundred and thirty-eight accessions of Shantung maple that grow naturally in fourteen regions were collected from nine provinces of China during the months of October and November, 2016 (Table 1 and Figure 1). Approximately ten individual disease-free, insect pest-free adult plants (each 20 years of age or older) were selected from each sample collection area. In order to minimize other factors influencing seed development, individual trees were chosen with spacing to other trees of at least 50 m. For each accession ~1 to 2 kg of fully matured samaras were picked randomly from numerous positions on each tree to ensure the samples were representative of each entire plant. Samaras were stored at room temperature and each was divided into two parts; the seed of one part was removed from its seed coat for analysis of oil content and fatty acid components, while the other part was used for planting. By using GPS to record latitude, longitude and elevation, meteorological factors were listed using local meteorological department data (Table 1).

Oil content and fatty acid analysis

Seed oil content of each of the 138 accessions was estimated using a Bruker minispec mq20 pulsed nuclear magnetic resonance instrument (pulsed NMR). The specific methods used followed official standard methods (ISO 5511:1992, GB/T 15690-1995;

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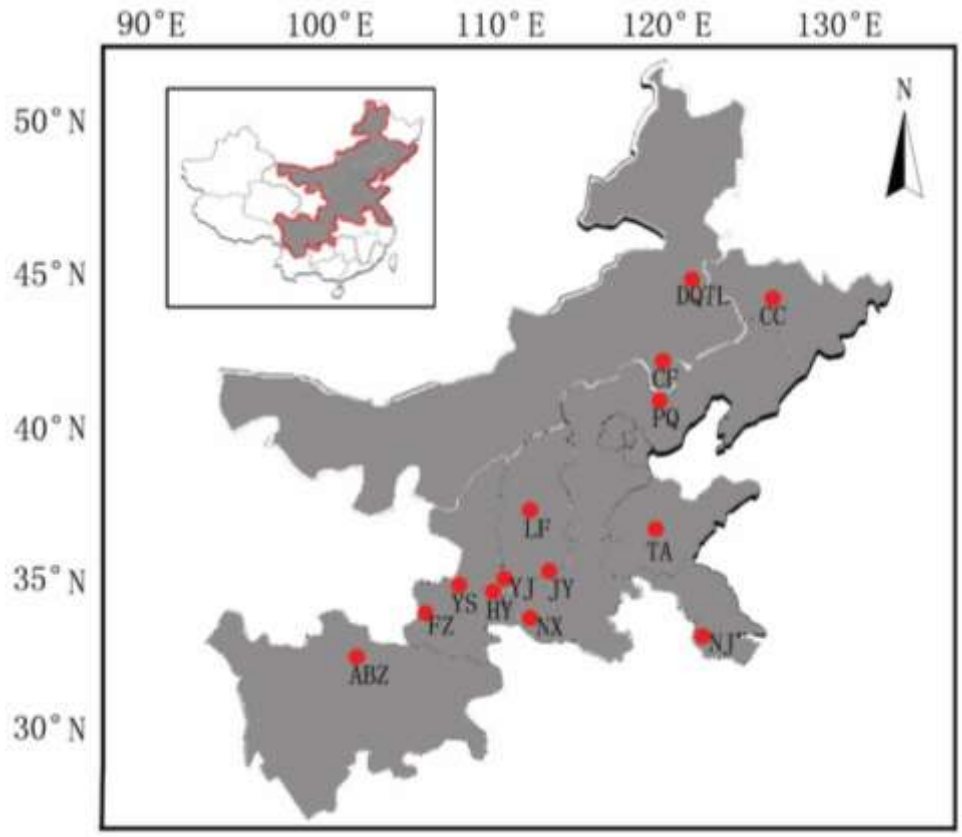

Figure 1. Map of the sampling area of Shantung maple showing sampling area, codes listed in Table 1.

AOAC, 2005). Oil extraction was performed using a Soxhlet apparatus with ~5 g of ground seeds and petroleum ether (60 to 90°C) solvent following published methods (Hu et al., 2017). The pure seed oil was transferred into a small glass vial, flushed with nitrogen and maintained at -20°C until further analysis. Seed oil was methylated twice, the first step is pre-esterification with H_2SO_4-CH_3OH to reduce the acid value to below 1 mg KOH/g, the second step is trans-esterification with KOH-CH_3OH according to the published method (Wei et al., 2008; ISO 12966-2, GB/T 17376-2008). The fatty acid methyl esters (FAMEs) profiles obtained for each accession were determined using an Agilent 7890A (Agilent, Palo Alto, CA, USA) gas chromatograph (GC) equipped with a flame ionization detector (FID) using 17:0 FAME as an internal standard. The DB-23 capillary column (length 30 m, inner diameter 0.32 mm, film thickness 0.25 μm) was used in this detection. The injector and detector temperatures were 230 and 280°C, respectively. Oven temperature was held at 180°C for 5 min, with a rise of 3°C min^{-1} to 230°C. The carrier gas (helium) was delivered using a flow rate of 1.0 ml min^{-1} and 1 μl samples were injected manually using a split injection mode. Peaks of fatty acid methyl ester peaks were identified by comparing their retention times with those of known standards run under the same conditions. Peak integration was performed using instrument software.

Biodiesel properties

Saponification number (*SN*) and iodine value (*IV*) were calculated from the FAME composition profile of the oil using Equations 1 and (2) (Kalayasiri et al., 1996):

$$SN = \sum \frac{560 \times A_i}{MW_i} \quad (1)$$

$$IV = \sum \frac{254 \times DB \times A_i}{MW_i} \quad (2)$$

where A_i represents the percentage of ith FAME, *DB* is the number of double bonds and *MWi* designates the molecular mass of each component. The cetane number (*CN*) of oil was calculated using Equation 3 (Krisnangkura, 1986):

$$CN = 46.3 + 5458 / SN - 0.225 \times IV \quad (3)$$

The physical properties of FAMEs, such as density (*D*) and kinematic viscosity (*v*), were calculated using equation (4) (Ramírez-Verduzco et al., 2012):

$$f_b = \sum_{i=1}^{n} A_i . f_i \quad (4)$$

where f_b is a function representing physical properties (*D* or *v*) and f_i is a function of the individual ith FAME properties (*D* or *v*).

The biodiesel-relevant traits of 138 *A. truncatum* accessions were compared with standard recommended values ASTM D6751

Table 1. *Acer truncatum* collection regions with respective designation codes and collection site characteristics.

Code	Number of accessions	Collection site	Latitude (°N)	Longitude (°E)	Altitude (m)	Annual average temperature (°C)	Annual rainfall (mm)	Frost-free season (d)
DQTL	10	Daiqintala, Inner Mongolia	45°13'	121°30'	324	5.6	388.0	120
CF	10	Chifeng, Inner Mongolia	42°17'	118°59'	574	7.4	460.0	130
CC	10	Changcun,Jilin	43°53'	125°19'	225	4.8	580.0	150
PQ	10	Pingquan,Hebei	40°50'	118°46'	628	6.0	600.0	155
TA	10	Taian,Shandong	36°12'	117°7'	305	13.2	722.6	202
LF	10	Linfen,Shanxi	36°44'	111°48'	802	10.0	625.0	153
YJ	10	Yongji,Shanxi	34°50'	110°22'	316	14.1	530.0	219
HY	10	Huayin,Shaanxi	34°32'	110°05'	353	12.0	600.0	200
YS	10	Yongshou,Shaanxi	34°43'	108°03'	1005	13.2	578.6	205
FZ	10	Fengzhou,Shaanxi	33°58'	106°39'	1020	11.4	613.2	188
ABZ	10	Abazhou,Sichuan	33°16'	103°55'	2060	12.7	552.9	225
NX	10	Neixiang,Henan	33°3'	110°51'	160	15.1	855.6	227
JY	10	Jiyuan,Henan	35°9'	112°7'	602	14.6	860.0	220
NJ	8	Nanjing,Jiangsu	32°15'	119°8'	50	15.4	1106.0	237

(2010), EN 14214 (2008) and GB/T 20828 (2015).

Statistical analysis

Analysis of variance (ANOVA) and correlation analysis were performed using SPSS 22.0 software (IBM). Other calculations were conducted using Excel 2010. The average for all plants in a sample collection location was used as the value for that region. Determinations were run in duplicate and the data were reported as the mean

RESULTS AND DISCUSSION

Oil content

Oil content is an important indicator of efficient biodiesel production because initially high oil content ultimately reduces overall production costs (Kumar and Sharma, 2011). In this study, highly distinct differences in seed oil content were observed among trees grown in the 14 sampling regions studied (Table 2). The highest seed oil content was collected from DQTL (32.47%), followed by YS (32.09%), and with the lowest level of 24.06% recorded in NJ. Therefore, DQTL and YS were chosen for subsequent screening of germplasms with high oil content. Among the 138 germplasm accessions tested, oil content ranged from 17.81 to 36.56%, with a mean value of 28.57% (Table 2). Moreover, oil content levels of most accessions were much higher than for *Glycine max* (17%), *Olea europaea* (20%) and *Sapium sebiferum* (12 to 29%) (USDA, 2012; Karmakar et al., 2010) and were comparable to levels for *Jatropha curcas* (20.05 to 38.33%) (Kaushik and Bhardwaj, 2013) and wild Manihot spp. (17 to 31%) (Alves et al., 2014). In YS-6 (36.56%) and DQTL-8 (35.44%) accessions studied here, oil content levels were greater than 35% (Table 3). These Shantung maple accessions thus hold promise to facilitate future development of lines with higher oil content.

Fatty acid composition

Seed oil quality and utility largely depend on fatty acid composition (Harrington, 1986). Fatty acids exhibit rich variety, exhibiting variable fatty acid composition and content across species (and even across varieties). Therefore, fatty acid composition and content profiles can be used as fingerprints to identify useful biological resources, in addition to their current use for oil authentication (Li et al., 2011). At the species level, a total of 15 distinct fatty acid components

Table 2. Variability in oil content and fatty acid composition for trees grown in 14 regions of Shantung maple.

Sampling regions		Fatty acid composition (%)															Oil content/%	*O/L*	*PUFA/ MUFA*	*C20-24/ C16-18*
		C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0	C22:1	C24:0	C24:1				
Between populations	DQTL	4.38	0.11	0.07	0.04	2.38	25.64	35.08	2.62	0.25	8.06	0.27	0.72	15.14	0.27	4.97	32.47	0.73	0.70	0.42
	XF	4.52	0.12	0.08	0.05	2.30	24.06	35.64	2.96	0.24	7.92	0.31	0.75	15.26	0.32	5.47	28.97	0.67	0.74	0.43
	CC	4.29	0.13	0.07	0.05	2.29	24.70	33.44	2.84	0.24	7.80	0.27	0.77	16.50	0.36	6.23	30.02	0.72	0.65	0.47
	PQ	4.47	0.06	0.04	0.03	2.37	22.94	34.44	2.90	0.27	7.43	0.30	0.85	17.23	0.38	6.29	30.38	0.66	0.70	0.49
	TA	4.93	0.12	0.07	0.07	2.21	27.91	31.34	2.37	0.25	8.20	0.26	0.73	15.40	0.30	5.85	26.71	0.88	0.59	0.45
	LF	4.70	-	-	-	2.45	26.64	30.30	2.79	0.28	8.31	0.29	0.88	16.90	0.43	6.03	29.03	0.87	0.57	0.50
	YJ	4.51	-	-	-	2.20	24.72	33.41	2.87	0.27	7.83	0.29	0.91	17.04	0.41	5.54	25.38	0.73	0.66	0.48
	HY	4.57	-	-	-	2.15	25.63	32.76	2.40	0.28	8.11	0.29	0.83	16.77	0.38	5.82	30.37	0.77	0.63	0.48
	YS	4.54	-	-	-	2.26	25.34	33.19	3.12	0.28	8.07	0.28	0.82	16.26	0.34	5.52	32.09	0.76	0.66	0.46
	FZ	4.92	0.14	0.11	0.09	2.84	23.46	34.16	2.55	0.31	7.72	0.32	0.94	16.97	0.34	4.91	27.36	0.68	0.69	0.46
	ABZ	5.06	0.17	0.08	0.02	2.69	23.30	34.94	3.10	0.29	7.34	0.31	0.86	16.27	0.31	5.24	31.70	0.66	0.73	0.44
	NX	4.90	0.16	0.06	0.06	1.59	26.32	30.61	2.87	0.26	7.75	0.24	1.01	17.50	0.50	6.18	25.39	0.85	0.58	0.50
	JY	5.14	0.14	0.06	0.01	2.30	24.93	31.41	2.98	0.28	7.92	0.31	0.86	16.72	0.41	6.54	25.09	0.79	0.62	0.49
	NJ	4.67	0.07	0.05	0.02	2.07	27.43	30.41	2.12	0.29	8.26	0.27	0.95	16.95	0.42	6.06	24.06	0.89	0.55	0.50
	F	5.302**	24.268**	22.226**	17.784**	17.244**	5.342**	13.814**	2.983**	3.006**	6.382**	2.194*	10.634**	7.220**	10.180**	7.740**	6.703**	7.346**	11.725**	10.681**
	Mean	4.69	0.09	0.05	0.03	2.30	25.19	32.97	2.76	0.27	7.90	0.29	0.85	16.49	0.37	5.76	28.57	0.77	0.65	0.47
Between the individual	Range	3.78-6.12	0-0.29	0-0.20	0-0.13	1.31-3.30	20.00-34.31	27.08 - 36.71	1.60-4.35	0.19-0.40	6.48-9.15	0-0.43	0.58-1.17	13.64-18.86	0.18-0.60	3.90-7.85	17.81-36.56	0.55-1.26	0.46-0.79	0.38-0.55
	CV	9.12	84.38	84.78	111.50	15.15	9.58	6.95	20.58	15.50	5.59	17.87	13.53	6.70	22.02	12.62	14.72	16.29	11.54	7.16

* indicates a significant difference at the 0.05 level; ** indicates an extreme significant difference at the 0.01 level; - indicates that it is not detected.

were detected in this study (Table 2), the results are similar to Hu et al. (2017), and a number similar to that was obtained for X. sorbifolia by Yu et al (2017), although their oil content differed from values reported here. Unsaturated fatty acids mainly include oleic acid (C18:1) (25.19%), linoleic acid (C18:2) (32.97%), linolenic acid (C18:3) (2.76%), cis-11-eicosenoic acid (C20:1) (7.90%), erucic acid (C22:1) (16.49%), nervonic acid (C24:1) (5.76%) and small amounts of Palmitoleic acid (C16:1) (0.09%), heptadecenoic acid (C17:1) (0.03%), and cis-11,14-eicosadienoic acid (C20:2) (0.29%). Saturated fatty acids mainly included palmitic acid (C16:0) (4.69%), stearic acid (C18:0) (2.30%) and small amounts of heptadecanoic acid (C17:0) (0.05%), arachidic acid (C20:0) (0.27%), behenic acid (C22:0) (0.85%), and tetracosanoic acid (C24:0) (0.37%). Indeed, fatty acids with content less than 0.5% exhibited high coefficients of variation (*CV* > 15%); conversely, the *CV* values of fatty acids with high oil content (for example oleic acid, linoleic acid and others) were low (< 15%), indicating that the fatty acid composition of ATO has certain stability. However, individual fatty acids were not present in all Shantung maple accessions and sampling regions. For example, palmitoleic acid, heptadecanoic acid and heptadecenoic acid were not detected in LF, YJ, HY and YS regions.

In this study, apart from cis-11,14-eicosadienoic acid (C20:2), the remaining fatty acids exhibited extremely significant difference ($p < 0.01$) among

Table 3. Statistical values of some accessions mentioned in the text.

Code	Fatty acid composition (%)															Oil content (%)	*O/L*	*PUFA/ MUFA*	*C20-24/C16-18*	Fuel properties				
	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0	C22:1	C24:0	C24:1					*SN*	*IV*	*CN*	*D*	*v*
DQTL-1	4.58	0.11	0.08	0.04	2.50	26.64	35.40	2.07	0.26	8.06	0.26	0.73	14.26	0.25	4.76	33.82	0.75	0.70	0.40	182.48	109.58	51.55	877.73	4.98
DQTL-6	4.56	0.09	0.08	0.06	2.50	25.88	36.71	2.43	0.25	8.19	0.27	0.73	13.64	0.28	4.32	32.03	0.70	0.76	0.38	182.84	111.53	51.06	878.00	4.94
DQTL-8	4.36	0.10	0.07	0.04	2.27	25.50	34.66	2.56	0.27	8.16	0.28	0.74	15.50	0.29	5.20	35.44	0.74	0.69	0.44	181.87	109.90	51.58	877.75	5.03
TA-10	4.77	0.11	0.06	0.06	2.02	34.31	27.13	1.60	0.19	8.82	0.17	0.58	15.16	0.18	4.84	28.83	1.26	0.46	0.43	181.98	101.84	53.38	876.67	5.08
YS-6	4.61	-	-	-	2.07	26.48	32.33	2.96	0.29	8.26	0.26	0.88	15.65	0.38	5.82	36.56	0.82	0.63	0.46	181.43	108.20	52.04	877.51	5.09
CF-2	3.99	0.15	0.07	0.05	2.40	21.86	36.36	3.84	0.23	8.22	0.38	0.79	15.23	0.36	6.07	29.84	0.6	0.79	0.46	181.61	113.70	50.77	878.26	5.04
CF-3	4.57	0.15	0.08	0.05	2.51	24.81	35.71	3.48	0.25	8.34	0.32	0.69	14.16	0.27	4.62	31.82	0.69	0.76	0.40	182.62	112.41	50.89	878.12	4.96
CF-9	4.61	0.12	0.10	0.04	2.24	23.36	35.57	3.95	0.25	7.63	0.32	0.76	15.19	0.33	5.54	29.87	0.66	0.77	0.43	182.02	112.92	50.88	878.17	5.01

- indicates that it is not detected.

the sample collection regions. Moreover, the differences between fatty acids among individual accessions were very obvious (Table 2), which had a wide range. Especially oleic acid and linoleic acid were the most prominent (20.00 to 34.31% and 27.08 to 36.71%, respectively). These differences in the fatty acid profile may be due to the variations between environmental conditions as well as genetic background (Zubr and Matthäus, 2002).

The stability index has previously been defined as the ratio of oleic to linoleic acid content (*O/L*) (Sorkheh et al., 2016) and the O/L ratio is an important factor determining the maximum storage time of ATO. In this study, oleic acid and linoleic acid were the most abundant fatty acids in seed oil, ranging from 20 to 34.31% and 27.08 to 36.71%, respectively (Table 2); the sum total of the averages of these two fatty acids was about 58% of the total fatty acid content. In this study, the O/L ratio varied from 0.55 to 1.26 among the various accessions, with a high CV (16.29%) (Table 2). O/L variances among sampling regions NJ (0.89), TA (0.88) and LF (0.87) were obviously higher than among other regions (Table 2). Meanwhile, thermo-oxidation resistance and low solidification point as good tribological properties of oils are characterized by low C20-24/C16-18 and low PUFA/MUFA ratios (Rodríguez-Rodríguez et al., 2013).

In this study, PUFA/MUFA ratios ranged from 0.46 to 0.79 and the C20-24/C16-18 ratios ranged from 0.38 to 0.55 (Table 3). The *CV*s of these two indexes were low (less than 15%), indicating that these traits were relatively stable. Accession TA-10 exhibited the lowest PUFA/MUFA ratio (0.46), while DQTL-6 exhibited the lowest C20-24/C16-18 ratio of all 138 accessions (0.38) (Table 3). Meanwhile, samples from TA collection region exhibited both low PUFA/MUFA ratio and low C20-24/C16-18 ratio (Table 2), indicating low solidification point and anti-oxidation ability.

Biodiesel properties

Saponification number (*SN*), iodine value (*IV*), cetane number (*CN*), density (*D*) and kinematic viscosity (*v*) were used to predict the utility of FAMEs as a biodiesel resource (Azam et al., 2005). The five calculated biodiesel properties varied between different Shantung maple accessions (Table 4).

SN represents the number of milliliters of KOH consumed during neutralization and saponification of 1 g of oil under specified conditions. It is a measure of the average molecular weight of all of the fatty acids present in oil. A higher *SN* correlates with a lower fatty acid molecular weight, stronger fluidity and greater potential for biodiesel use. *SN* ranged from 180.26 to 182.86, with a mean value of 181.33 among the 138 accessions, these calculated values are slightly lower than the previous report (Wang, 2013) (185 to 190). From the point of view SN, FAME and ATO is suitable for biodiesel production.

IV is a measure of oil unsaturated fatty acid levels, a higher *IV* indicates that more C=C bonds are present in the oil (Moser, 2011). In general, biodiesel must contain a certain proportion of unsaturated fatty acids, with *IV* increasing with increasing unsaturated fatty acid content. However, biodiesel with too high an *IV* level will lead to polymerization of unsaturated bonds during the combustion process, resulting in decreased engine lubrication (Yu et al., 2017). Therefore, the biodiesel standard EN 14214 limits *IV* to a maximum value of 120. Here, the *IV*

Table 4. Biodiesel properties of Shantung maple seed oil methyl esters with comparison to biodiesel standards.

Parameter	Range		Average±SD	*CV*	Standards		
	max	min			ASTM D6751-10	EN 14214-08	GB/T 20828-07
Saponification number	182.86	180.26	181.33±0.59	0.33	-	-	-
Iodine value (g I_2 /100g)	113.70	101.84	108.11±2.61	2.41	-	120 max	-
Cetane number	53.53	50.77	52.08±0.63	1.22	47 min	51 min	49 min
Density(kg/m^3 ; 20°C)	880.08	873.03	877.40±0.78	0.09	-	860-900	820-900
Kinematic viscosity (mm ^{2}/ s ;40°C)	5.28	4.92	5.10±0.06	1.24	1.9-6.0	3.50-5.00	1.9-6.0

- No specified limit.

ranged from 101.84 to 113.70, with a mean value of 108.11 among the 138 accessions, consistent with the report by Wang (2013) (100 to 110), indicating that ATO is a semidrying type and all individual accessions met standard *IV* requirements. Meanwhile, the concentration of linolenic acid and acids containing four double bonds is also in line with European standard organization that FAMEs should not exceed the limit of 12 and 1%, respectively (UNE-EN 14214, 2008).

CN is an important indicator that characterizes the spontaneous combustion performance of the fuel in a diesel engine. *CN* influences engine operation, wear, emissions and noise and itself is influenced by the selection of methyl esters. The *CN* of biodiesel depends on the length of the carbon chains and the number of unsaturated double bonds (Knothe et al., 2003). A fuel with an appropriate CN has a short stagnation period and high heat utilization efficiency. *CN* is prescribed as a minimum value of 47 in the biodiesel standard ASTM D6751-10, 51 in EN 14214-08 and 49 in GB/T 20828-07.

In this study, CN values ranged from 50.77 to 53.53, with a mean value of 52.08 among the 138 accessions. Therefore, all of the CN values obtained here satisfied specifications outlined in biodiesel standards ASTM D6751-10 and GB/T 20828-07, and met the vast majority of recommended values outlined in standard EN 14214-08 (except for CF-2, CF-3 and CF-9; values of 50.77, 50.89 and 50.88, respectively (Table 3). The selection and cultivation of Shantung maple germplasms, especially with higher *CN* are urgent in future.

D and *v* have direct effects on the atomization process during combustion (Ramírez-Verduzc et al., 2012). *D* values are limited to ranges of 860 to 900 and 820 to 900, as specified by the EN 14214-08 and GB/T 20828-07 standards, respectively. In this work, *D* varied from 873.03 to 880.08 among the 138 accessions and thus met the requirements outlined in the standards listed directly above. Moreover, the *v* values of the 138 accessions varied from 4.92 to 5.28 and thus all met the requirements set forth in standards ASTM D6751-10 (1.9-6.0), and GB/T 20828-07 (1.9-6.0), but the vast majority did not meet the standard EN 14214-08 (3.50 to 5.00).

The *CV*s of these five parameters, *SN*, *IV*, *CN*, *D* and *v*, were each less than 2.5% (with values of 0.33, 2.41, 1.22, 0.09 and 1.24%, respectively) demonstrating that the differences between accessions were small and the traits were thus stable even if the fatty acid component of the seed oil has a large difference. Moreover, these results are superior to published results for *X. sorbifolia* (Yu et al., 2017), *J. Curcas* (Sinha et al., 2015) for several recommended diesel energy parameters. From the aforementioned analyses, it can be seen that the main physical and chemical properties of FAMEs meet biodiesel industry standards ASTM D6751-10 and GB/T 20828-07, indicating that Shantung maple can be regarded as a non-grain diesel oil crop with great potential for further biodiesel development. So, this is feasible through the cultivation of Shantung maple in the barren land, which does not affect the development of agricultural industry, but also ease the demand for petroleum.

Correlation among traits

Correlation analysis showed a significant and negative correlation of oil content with palmitic acid (-0.382, $p<0.01$) and oleic acid (-0.195, $p<0.05$), while stearic acid (0.254, $p<0.01$) and linoleic acid (0.340, $p<0.01$) exhibited a significant positive correlation with oil content (Table 5).

Table 5. Correlation coefficients among seed oil content, fatty acid content and biodiesel properties of Shantung maple accessions.

Trait	C16:0	C18:0	C18:1	C18:2	C18:3	C20:1	C22:1	C24:1	Oil content	*SN*	*IV*	*CN*	*D*	*v*
C16:0	1													
C18:0	0.054	1												
C18:1	-0.123	-0.268**	1											
C18:2	-0.088	0.338**	-0.766**	1										
C18:3	0.048	0.105	-0.520**	0.316**	1									
C20:1	-0.193*	-0.078	0.698**	-0.439**	-0.288**	1								
C22:1	0.024	-0.215*	-0.290**	-0.266**	-0.046	-0.478**	1							
C24:1	0.012	-0.475**	-0.203*	-0.275**	0.103	-0.336**	0.583**	1						
Oil content	-0.382**	0.254**	-0.195*	0.340**	0.163	-0.003	-0.111	-0.165	1					
SN	0.105	0.312**	0.136	0.418**	0.082	0.295**	-.891**	-0.767**	0.156	1				
IV	-0.210*	0.201*	-0.715**	0.915**	0.610**	-0.366**	-0.312**	-0.200*	0.386**	0.421**	1			
CN	0.178*	-0.234**	0.640**	-0.911**	-0.577**	0.293**	0.426**	0.303**	-0.381**	-0.543**	-0.990**	1		
D	-0.163	-0.252**	-0.121	0.260**	0.298**	-0.032	-0.221**	0.117	0.171*	0.334**	0.415**	-0.436**	1	
v	0.025	-0.408**	0.131	-0.662**	-0.221**	-0.122	0.811**	0.777**	-0.278**	-0.906**	-0.660**	0.750**	-0.149	1

*p<0.05; **p<0.01

These results reflect the fact that germplasms with high oil content accumulate crude fat mainly through transformation of oleic acid to linoleic acid via desaturation; this process is influenced by the initial content of individual fatty acids in oil.

Meanwhile, correlation analysis among other fatty acid components revealed that the highest positive correlation was observed between oleic acid and cis-11-eicosenoicacid (0.698, p<0.01), while the highest negative correlation was found between oleic acid and linoleic acid (-0.766, p<0.01). This association is well documented and has been reported for other oil crops, including sesame (Were et al., 2006), soybean (Patil et al., 2007), and wild almond (Sorkheh et al., 2016). Oleic acid is considered to be the precursor of PUFAs. This transformation can occur via two pathways: one pathway produces linoleic acid and linolenic acid under the action of enzymes FAD 2 and FAD 3, while the other produces cis-11-eicosenoicacid, erucic acid and nervonic acid by extending the carbon chain (Sayanova et al., 1997). In ATO, cis-11-eicosenoicacid acid showed significant (p<0.01) and negative correlations with both linoleic acid (-0.439) and linolenic acid (-0.288). This observation suggests that the two transformation processes are competitive. Therefore, suppression of one of the competing processes could facilitate generation of fatty acids produced by the other transformation process. Such a strategy has been successfully used in *Brassica carinata* (Katavic et al., 2010). In this study linoleic acid showed a significant positive correlation with linolenic acid (p<0.01, 0.316), while cis-11-eicosenoicacid showed a significant (p<0.01) negative correlation with erucic acid (-0.478) and nervonic acid (-0.336). Concurrently, significant (p<0.01) positive correlations were observed between erucic acid and nervonic acid (0.583).

SN showed a significant positive correlation (p<0.01) with *IV* (0.421) and *D* (0.334) while a significant negative correlation (p<0.01) with *CN* (-0.543) and *v* (-0.906). *IV* showed a significant positive correlation (p<0.01) with *D* (0.415) and a significant negative correlation (p<0.01) with *CN* (-0.990) and *v* (-0.660). *CN* showed a significant positive correlation (p<0.01) with *v* (0.750) and a significant negative correlation (p<0.01) with *D* (-0.436). It is know that *CN* is the ability of fuel to ignite quickly after being injected and a higher value indicates better ignition quality of fuel (Sorkheh et al., 2016). From the 3.3 analysis, it can be seen that with the appropriate increase in *CN*, the other four biodiesel properties still meet the standard. Correlation studies showed a significant (p<0.01) and positive correlation with C18:1, C20:1, C22:1 and C24:1, while C18:2 and C18:3 showed a significant (p<0.01) negative correlation with *CN*. Therefore, it is effective to

choose the accession with high monounsaturated fatty acid (MUFA) content as optimal germplasm resources for biodiesel production.

Conclusion

Calculations showed that produced FAMEs of ATO meet or exceed biodiesel fuel requirements. Thus, Shantung maple is an energy tree that holds great promise for use in biodiesel production and correlation studies showed that the accession with high MUFA content is suitable as optimal germplasm resources for biodiesel production. Geographic locations of DQTL, YS and TA were demonstrated to be optimal Shantung maple farming regions, which should be first considered in building plantations for biodiesel production. In addition, DQTL-1, DQTL-6, DQTL-8, YS-6, and TA-10 were demonstrated to be optimal biodiesel germplasm resources, exhibiting excellent seed oil content and fatty acid composition profiles. Furthermore, this study enriches the fatty acid database of woody oil species and establishes quality criteria for screening Shantung maple genotypes for development of optimal germplasm for biodiesel production purposes. Ultimately, this work may guide the development of more effective strategies for biodiesel production to help address the world's growing energy needs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATION

ATO, *A. truncatum* seed oil; **FAMEs,** fatty acid methyl esters; **GC,** gas chromatograph; **FID,** flame ionization detector; ***SN***, saponification number; ***IV,*** iodine value; ***CN,*** cetane number; ***D,*** density; ***V,*** kinematic viscosity; **ANOVA,** analysis of variance; **C16:0,** palmitic acid; **C16:1,** palmitoleic acid; **C17:0,** heptadecanoic acid; **C17:1,** heptadecenoic acid; **C18:0,** stearic acid; **C18:1,** oleic acid; **C18:2,** linoleic acid; **C18:3,** linolenic acid; **C20:0,** arachidic acid; **C20:1,** cis-11-eicosenoic acid; **C20:2,** cis-11,14-eicosadienoic acid; **C22:0,** behenic acid; **C22:1,** erucic acid; **C24:0,** tetracosanoic acid; **C24:1,** nervonic acid; ***CV,*** coefficients of variation; ***O/L,*** oleic to linoleic acid content; ***PUFA/MUFA,*** polyunsaturated fatty acids/monounsaturated fatty acids; **MUFA,** monounsaturated fatty acids; **PUFA,** polyunsaturated fatty acids.

REFERENCES

Akoh CC, Moussata CO (2001). Characterization and oxidative stability of enzymatically produced fish and canola oil-based structured lipid. J. Am. Oil Chem. Soc. 7(1):25-30.

Alves AAC, Manthey L, Isbell T (2014). Diversity in oil content and fatty acid profile in seeds of wild cassava germplasm. Ind. Crop. Prod. 60:310-315.

Association of Official Analytical Chemists (AOAC) (2005). Official methods of analysis of AOAC international. Gaithersburg: AOAC International.

Azam MM, Waris A, Nahar NM (2005). Prospects and potential of fatty acid methyl esters of some non-traditional seed oils for use as biodiesel in India. Biomass Bioenergy 29:293-302.

Evans DA, Bennett DA, Wilson RS, Bienias JL, Morris MC, Scherr PA (2003). Incidence Alzheimer disease in a biracial urban community: Relation to apolipoprotein E allele status. Neurol. Res. 60(2):185-189.

GB/T 15690 (2008). Oil seeds - Detemination of oil content - Method using continuous-wave low -resolution nuclear magnetic resonance spectrometry (Rapid Method). http://down.foodmate.net/standard/sort/3/27403.html

GB/T 17376 (2008). Animal and vegetable fats and oils - Preparation of methyl esters of fatty acids. http://down.foodmate.net/standard/sort/3/17445.html

Harrington KJ (1986). Chemical and physical properties of vegetable oil esters and their effect on diesel fuel performance. Biomass 9:1-17.

Honma A, Koyama T, Yazawa K (2010). Anti-hyperglycemic effects of sugar maple *Acer saccharum* and its constituent acertannin. Food Chem. 123:390-394.

Hu P, Xu XB, Yu LL (2017). Physicochemical Properties of *Acer truncatum* Seed Oil Extracted Using Supercritical Carbon Dioxide. J. Am. Oil Chem. Soc. 94: 779-786.

Karmakar A, Karmakar S, Mukherjee S (2010). Properties of various plants and animals feedstocks for biodiesel production. Bioresour. Technol. 10:7201-7210.

Kalayasiri P, Jayashke N, Krisnangkura K (1996). Survey of seed oils for use as diesel fuels. J. Am. Oil Chem. Soc. 73:471- 474.

Katavic V, Mietkiewska E, Taylor DC, Guo YM, Brost JM (2010). *Lunaria annua*,*cardamine graeca* and *teesdalia nudicaulis* fae Genes and their Use in Producing Nervonic and Eicosenoic Acids in Seed Oils. Patent Application Publication. No.US 2010/0122377.

Kaushik N, Bhardwaj D (2013). Screening of *Jatropha curcas* germplasm for oil content and fatty acid composition. Biomass Bioenergy 58:201-218.

Krisnangkura K (1986). A simple method for estimation of Cetane index of vegetable oil methyl esters. J. Am. Oil Chem. Soc. 63: 552-553.

Knothe G, Matheaus AC, Ryan III TW (2003). Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester. Fuel 83:971-975.

Kumar A, Sharma S (2011). Potential non-edible oil resources as biodiesel feedstocks: An Indian perspective. *Renew. Sust.* Energy Rev. 15:1791-1800.

Li CM, Yao YP, Zhao GZ, Cheng W, Liu H, Liu C, Shi Z, Chen Y, Wang S (2011). Comparison and analysis of fatty acids, sterols and tocopherols in eight vegetable oils. J. Agric. Food. Chem. 59:12493-12498.

Li L, Manning WJ, Tong L, Wang XK (2015). Chronic drought stress reduced but not protected Shantung maple (*Acer truncatum* Bunge) from adverse effects of ozone (O_3) on growth and physiology in the suburb of Beijing, China. Environ. Pollut. 201:34-41.

Liu XY, Wang SX (2009). Preparation of biodiesel from *Acer truncatum*

oil. Yunnan Chem. Technol. 36(1): 9-11.
Lovato L, Pelegrini BL, Rodrigues J, Braz de Oliveira AJ, Piloto Ferreira IC (2014). Seed oil of *Sapindus saponaria* L. (Sapindaceae) as potential C16 to C22 Fatty acids resource. Biomass Bioenergy 60:247-251.
Ma X, Wu L, Ito Y, Tian W (2005). Application of preparative high-speed counter current chromatography for separation of methyl gallate from *Acer truncatum* Bunge. J. Chromatogr. A 1076:212-215.
Moser BR (2011). Influence of extended storage on fuel properties of methyl esters prepared from canola, palm, soybean and sunflower oils. Renew. Energy 36:1221-1226.
Mukta N, Murthy IYLN, Sripal P (2009). Variability assessment in *Pongamia pinnata* (L.) Pierre germplasm for biodiesel traits. Ind. Crops Prod. 29:536-540.
Patil A, Taware SP, Oak MD, Tamhankar SA, Rao VS (2007). Improvement of oil quality in soybean [*Glycine max* (L.) Merrill] by mutation breeding. J. Am. Oil Chem. Soc. 84:1117-1124.
Ramírez-Verduzco LF, Rodríguez-Rodríguez L, Jaramillo-Jacob AR (2012). Predicting cetane number, kinematic viscosity, density and higher heating value of biodiesel from its fatty acid methyl ester composition. Fuel 91:102-111.
Rodríguez-Rodríguez MF, Sánchez-García A, Salas JJ, Garcés R, Martínez-Force E (2013). Characterization of the morphological changes and fatty acid profile of developing *Camelina sativa* seeds. Ind. Crops Prod. 50:673-679.
Sayanova D, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, Shewry PR, Napier JA (1997). Expression of a borage desaturase cDNA containing an N - terminal cy-tochrome b5 domain results in the accumulation of high levels of ∆6 - desaturated fatty acids in transgenic tobacco. Proc. Natl Acad. Sci. USA 94:4211-4216.
Sinha P, Islam MA, Negi MS, Tripathi SB (2015). Changes in oil content and fatty acid composition in *Jatropha curcas* during seed development. Ind. Crops Prod. 77:508-510.
Sorkheh K, Kiani S, Sofo A (2016). Wild almond (*Prunus scoparia* L.) as potential oilseed resource for the future: Studies on the variability of its oil content and composition. Food Chem. 212:58-64.
Song XH, Li H, Li CY, Xu JW, Hu DM (2016). Effects of VOCs from leaves of *Acer truncatum* Bunge and Cedrus deodara on human physiology and psychology. Urban For. & Urban Gree. 19:29-34.
USDA, Agricultural Research Service (2012). USDA National Nutrient Database for Standard Reference, Release 25. Nutrient Data Laboratory, Home Page, http://www.ars.usda.gov/ba/bhncr/ndl
Wang XY, Wang SQ (2011). New resources food - *Acer truncatum* seed oil. China Oil Fats 36(9):56-59.
Wang XY (2013). Purpleblow maple of China. Northwest A & F University Press.
Wei Q, Ma XH, Mao H (2008). Technology optimization on making biodiesel from the seed oil of *Acer truncatum.* Transact. Chin. Soc. Agric. Machinery 39(10):199-223.
Werea BA, Onkwarea AO, Gudua S, Welander M, Carlssonb AS, (2006). Seed oil content and fatty acid composition in East African sesame (*Sesamum indicum* L.) accessions evaluated over 3 years. Field Crops Res. 97:254-260.
Yu HY, Fan SQ, Bi QX, Wang SX, Hu XY, Chen MY, Wang LB (2017). Seed morphology, oil content and fatty acid composition variability assessment in yellow horn (*Xanthoceras sorbifolium* Bunge) germplasm for optimum biodiesel production. Ind. Crops Prod. 97:425-430.
Zhao WH, Gao CC, Ma XF, Bai XY, Zhang YX (2007). The isolation of 1,2,3,4,6-penta-O-galloyl-beta-d-glucose from *Acer truncatum* Bunge by high-speed counter-current chromatography. J. Chromatogr. B. 850:523-527.
Zubr J, Matthäus B (2002). Effects of growth conditions on fatty acids and tocopherols in Camelina sativa oil. Ind. Crop. Prod. 15:155-162.

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Full Length Research Paper

Effect of Roselle (*Hibiscus sabdariffa*) and ginger (*Zingiber officinale*) as feed additives, on growth and haematology of *Clarias gariepinus* Juvenile

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The rebirth and use of medicinal plants in aquaculture has become necessary as the use of synthetic drugs and chemicals is been discouraged due to their aftermath effects on cultured organisms and aquatic environment. A 56-day study was conducted to assess the effects of Roselle and ginger as dietary additives, on growth and hematology of *Clarias gariepinus* juvenile. Total of 150 *C. gariepinus* juveniles (35.41±1.45 g) were assigned to five iso-nitrogenous diets as treatments having ginger and roselle added as additives at varying inclusion levels of 0.0, 2.0, 4.0, 2.0 and 4.0 g/100 g. Best growth performance was observed in 4.0 g ginger treated fish followed by 4.0 g roselle fed fish group, while 2.0 g roselle fed fish had the lowest growth performance. Significant changes ($p<0.05$) were observed in the haematology of *C. gariepinus* fed varying inclusion levels of ginger and roselle. Highest values for red blood cells (4.07±0.08), haemoglobin (11.61±0.57) and pack cell volume (34.33±0.88) were seen in 4.0 g ginger treatment group followed by the control (3.63±0.22, 9.93±0.92 and 30.33±2.73), respectively. No significant changes were observed in red blood cells indices (mean corpular volume, mean corpular hemoglobin and mean corpular hemaglobin concentration). The current study revealed that fish fed 4.0 g ginger diet had better growth and haematological profile.

Key words: Phytobiotics, growth, hematology, *Clarias gariepinus*.

INTRODUCTION

Aquaculture expansion and development in developing countries has been hampered by various factors which includes environmental degradation and reduced water quality, underdeveloped credit markets, conflicts over the

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use of land and water resources, lack of governance and regulation in production, poorly developed infrastructure, limited access to fish seed, feed, increased fish feed extraction from the world's ocean and diseases (World Bank, 2006). Disease which is one of the critical factors hampering aquaculture development ensued as a result of negative interactions between fish and pathogenic bacteria (Romero et al., 2012; Iheanacho et al., 2017a).

Antibiotics are chemical compounds used in treating and preventing diseases in aquaculture. However, the use of antibiotics in fish feeds has a good impact on growth (Rico et al., 2013). Antibiotics growth promoters (AGP) were supposed to increase growth rate as a result of improved gut health, resulting in better nutrients utilization and improved feed conversion (Visek, 1978). But now, antibiotics growth promoters are considered as human health risk factor for their possible role in the emergence of microbial resistance, breakage of the animal intestinal micro-ecological balance and the presence of antibiotics residues in resultant fish products (Okeke and Ososa, 2003). Feed supplements are costly to the extent that most fish farmers depend on it to supplement some essential minerals and vitamins found wanting in fish feeds but currently, can no longer afford to use them.

Natural materials such as medicinal plants could be widely accepted as feed additives to enhance feed utilization and aquaculture productive performance and sustainability (Levic et al., 2008). Phytogenic feed additives, also known as phytobiotics products are plant derived products, used in animal feeding to improve performance through amelioration of feed properties, promotion of production performance and improving the quality of animal origin food/feed (Alector and Osho, 2009). World Health Organization encourages using medicinal herbs and plants to substitute or minimize the use of chemicals through the global trend of go back to nature (Adewole, 2014).

Roselle (*Hibiscus sabdariffa*) is an annual dicotyledonous, erect, herbaceous tropical plant. The plant is cultivated majorly in the northern part of Nigeria as edible vegetable and considered to be medicinal (Ijeomah et al., 2012). The chemical constituents of the flower include the flavonoids, gossypetine and sabdaretine (Pietta, 2000). Roselle is reported to be diuretic, digestive, antiseptic, sedative, purgative, emollient, demulcent and astringent (Adewole, 2014). The calyces have many medicinal applications in curing kidney stone, pyrexia, liver damage, hypertension and leukemia (Abu-Tarboush et al., 1997; Estrella et al., 2000).

Ginger (*Zingiber officinale*) is also a herbaceous perennial plant which grows annual stems about a meter tall, bearing narrow green leaves and yellow flowers (Kim et al., 2008). Ginger has been reported to be rich in mineral elements (magnesium, potassium, phosphorus, calcium and zinc), vitamins (retinol, cholecalciferol, ascorbic acid, thiamine, riboflavin, niacin, folic acid, pantothenic acid and pyridoxine) and phytochemicals (flavonoid and alkaloid) (USDA, 2014; Iheanacho et al., 2017a). Shubha (2015) reported that ginger rhizomes also contain gingerol, shogoal and a potent proteolytic enzyme called zingibain. The author also reported that ginger is anti-platelet, antibacterial, antifungal, antiviral, anti-worm, anti-inflammatory and has anti-oxidative activity.

The use of medicinal plants in aquaculture has not being widely practised. The current study seeks to evaluate the effects of roselle and ginger supplemented diets as feed additives, on growth and haematology of African catfish (*Clarias gariepinus*).

MATERIALS AND METHODS

Study area

The study was carried out at the Animal House of the Department of Microbiology/Biotechnology, Federal University, Ndufu Alike Ikwo, Ebonyi State, Nigeria.

Study fish

One hundred and fifty (150) *C. gariepinus* juveniles were procured from a private fish farm in Abakaliki and transported in a 50 L gallon filled with water to the Animal House of the Department of Microbiology/Biotechnology, Federal University, Ndufu Aliko Ikwo within 30 and 40 min. The fish were acclimated in a tarpaulin tank (4 m x 2 m x 1 m) for two weeks and were fed commercial fish feed (coppens) throughout the 2 weeks acclimation period.

Collection, preparation and processing of Roselle flower (*Hibiscus sabdariffa*) and ginger (*Zingiber officinale*)

The dried flower (calyx) of roselle and rhizomes of ginger were purchased from Abakpa Market located at Abakalik town, Ebonyi State, Nigeria. Both materials were ground into powder, using electric blender (Philips, model HRI701/BC, made in China), thus sieved, and stored separately in an air tight container until use.

Experimental diets

Five iso-nitrogenous diets were formulated to yield 37% crude protein (Table 1). Roselle and ginger were included in the diets as feed additives at varying inclusion levels and coded as HS1 and HS2 for Roselle, while G1 and G2 were for ginger. The different inclusions were HS1 (2.00 g), HS2 (4.00 g), G1 (2.00 g) and G2 (4.00 g). The control diet had 0% inclusion of both roselle and ginger. Other feed ingredients used in the diet formulation includes fish meal (FM) soy bean meal (SBM), wheat offal, yellow maize (YM), vitamin/mineral premix, cassava starch, palm oil, bone meal and salt (Table 1). Pearson square method was used in feed formulation. Samples of the experimental diets were sent to the laboratory for proximate analysis. Samples were analysed following the procedure of A.O.A.C (2000).

Experimental design

A total of one hundred and fifty fish (150) (initial weight 35.41±1.53 g) were randomly distributed to 15 aquarium plastic tanks (1 m x 1

Table 1. Percentage composition (g/100 g) of experimental diet.

Ingredient	Control	HS1	HS2	G1	G2
Fish meal	26.09	26.09	26.09	26.09	26.09
Soybean meal	21.74	21.74	21.74	21.74	21.74
Yellow maize	21.74	19.74	17.74	19.74	17.74
Wheat offal	21.74	21.74	21.74	21.74	21.74
Palm oil	1.96	1.96	1.96	1.96	1.96
Vit/min premix	1.52	1.52	1.52	1.52	1.52
Cassava starch	1.74	1.74	1.74	1.74	1.74
Salt	1.52	1.52	1.52	1.52	1.52
Bone meal	1.96	1.96	1.96	1.96	1.96
Roselle	0.00	2.00	4.00	0.00	0.00
Ginger	0.00	0.00	0.00	2.00	4.00
Total	100	100	100	100	100
Calculated crude protein (%)	37.00	37.00	37.00	37.00	37.00

m x 1 m) at ten fish per treatment. Each tank contained 30 L of water. Experimental diets were randomly assigned using completely randomized design to triplicate tanks. The fish were fed 5% body weight per day in two portions (by 9.00 am and 5.00 pm) for 8 weeks. Quantity of feed was adjusted forth nightly after batch-weighing of experimental fish. Water in the tanks was partly removed by siphoning and replaced with fresh water every three days to avoid fouling resulting from faeces and uneaten food. Water quality parameters such as temperature, dissolved oxygen and pH of the experimental water tanks were monitored and measured daily using water testing kits (PRO-LAB^{TM} Flourida). Temperature, pH and dissolved oxygen were maintained at 27.12±0.04°C, 6.98±0.01 and 6.05±0.21 mg L^{-1}, respectively.

Growth parameters

Weight measurement of the fish was obtained at the end of the experiment and the following growth parameters were determined according to the formula of Iheanacho et al. (2018): Mean weight gain (SWG); Specific growth rate (SGR); Food conversion ratio (FCR);

Mean weight gain (SWG) = final weight - initial weight

Specific growth rate (SGR)

$$SGR = \frac{(\text{Ln mean final weight} - \text{Ln mean initial weight}) \times 100}{\text{Time (days)}}$$

Ln = Natural logarithm

Food conversion ratio (FCR)

$$FCR = \frac{\text{Weight of food fed (Dry gram weight)}}{\text{Weight gain of fish (Wet gram weight)}}$$

Condition factor = K = $100W/Lb^3$ (Gomiero and Braga, 2005)

Where, K = condition factor; W = the weight of the fish in gram (g); L = the total length of the fish in centimetre (cm); b = the value obtained from the length-weight equation.

Haematological analysis

Three fish per tank were sampled for blood collection at the end of the experiment. Blood was collected from the caudal vein into an EDTA lithium tubes. Blood samples were immediately transported to the haematology laboratory unit of the Federal University, Ndufu Alike Ikwo Medical Centre for Haematological Analysis. The blood was analyzed to determine the packed cell value (PCV) with micro haematocrit using heparinised capillary tube (25 mm), while red blood cell (RBC), white blood cell (WBC) counts, haemoglobin (Hb) concentration and red cell indices (mean corpular volume (MCV), mean corpular hemoglobin (MCH) and mean corpular haemoglobin concentration MCHC) were determined as described by Dacie and Lewis (2011).

Statistical analysis

Data obtained from the experiment were subjected to one-way analysis of variance (ANOVA), using Statistical Package for Social Science (SPSS 2006, version 22). Duncan multiple range test (DMRT) was used to compare the differences between means at $p<0.05$. Data were presented as mean ± SE.

RESULTS

Results on proximate composition of the experimental diets are presented in Table 2. Highest percentages of crude protein (30.38±0.06), ash (8.21±0.02), dry matter (93.28±0.02) and fat (4.24±0.01) were seen in ginger (G2) based diet. Growth performance of *C. gariepinus* fed roselle and ginger supplemented diets are presented in Table 3. Significant difference ($p<0.05$) was observed among treated groups AS compared to the control.

Data on haematological responses of *C. gariepinus* fed roselle and ginger supplemented diets are presented in Table 4. Significant differences ($p<0.05$) were seen among treated groups as compared to the control.

Table 2. Proximate composition (%) of experimental diets.

Parameter	Control (0.00)	HS1(2.0 g)	HS2(4.0 g)	G1(2.0 g)	G2(4.0 g)
Dry matter	92.66±0.03^{e}	92.80±0.02^{d}	92.97±0.03^{c}	93.14±0.02^{b}	93.28±0.02^{a}
Crude protein	25.74±0.03^{e}	28.83±0.02^{d}	29.33±0.03^{c}	29.59±0.06^{b}	30.38±0.06^{a}
Ash	7.82±0.02^{e}	7.88±0.02^{d}	7.96±0.01^{c}	8.09±0.02^{b}	8.21±0.02^{a}
Crude fibre	2.35±0.01^{d}	2.56±0.01^{b}	2.52±0.01c	2.63±0.01^{a}	2.54±0.01^{bc}
Moisture	7.34±0.03^{a}	7.20±0.02^{b}	7.03±0.03^{c}	6.86±0.02^{d}	6.72±0.02^{e}
Fat	3.88±0.01^{e}	3.97±0.01^{d}	4.05±0.01^{c}	4.17±0.01^{b}	4.24±0.01^{a}
*NFE	52.88±0.01^{a}	49.58±0.01^{b}	49.12±0.01^{c}	48.67±0.04^{d}	47.92±0.05^{e}

* Nitrogen free extract (NFE) = (100 - (crude protein + ash+ crude fibre + moisture + fat)). Data presented represents mean values of parameters and values in the same rows with the same alphabet superscript are not significantly different (p>0.05).

Table 3. Growth response of *C. gariepinus* juvenile fed Roselle and ginger supplemented diets.

Parameter	Control (0.0 g)	HS1 (2.0 g)	HS29 (4.0 g)	G1 (2.0 g)	G2 (4.0 g)
Initial weight (g)	35.46±0.50^{a}	35.22±0.42^{a}	35.08±0.52^{a}	35.28±0.22^{a}	35.68±0.19^{a}
Final weight (g)	62.50±1.53^{ab}	53.03±1.24^{b}	63.95±2.54^{ab}	56.82±2.41^{ab}	67.51±0.44^{a}
Weight gain (g)	27.04±1.45^{ab}	17.82±1.58^{b}	28.88±6.07^{ab}	21.55±2.20^{ab}	31.82±0.63^{a}
SGR (%g/d)	2.74±0.01^{b}	2.73±0.01^{b}	2.73±0.01^{b}	2.73±0.01^{b}	3.13±0.21^{a}
FCR	1.02±0.20^{b}	1.24±0.15^{a}	1.18±1.25^{a}	1.12±0.34^{ab}	0.99±0.51^{b}
Condition factor (k)	0.61±0.08^{a}	0.39±0.05^{a}	0.45±0.02^{a}	0.43±0.00^{a}	0.60±0.11^{a}

Data represents mean ± (SE) values of parameters estimated. Values in the same rows with the same alphabet superscript are not significantly different (p>0.05).

Table 4. Hematological data of *C. gariepinus* fed Roselle and ginger supplemented diets.

Parameter	Control (0.0 g)	HS1 (2.0 g)	HS2(4.0g)	G1 (2.0 g)	G2 (4.0 g)
PCV (%)	30.33±2.73^{ab}	26.00±3.22^{ab}	25.67±0.88^{b}	28.00±3.46^{ab}	34.33±0.88^{a}
Hb (g.dL^{-1})	9.93±0.92^{a}	8.57±1.23^{a}	8.31±0.99^{a}	9.15±1.07^{a}	11.61±0.57^{a}
RBC(10^{12} L)	3.63±0.22^{ab}	3.08±0.43^{b}	3.27±0.09^{ab}	3.29±0.35^{ab}	4.07±0.08^{a}
WBC(10^{9} L)	4.30±0.27^{b}	6.50±0.38^{a}	5.99±0.26^{a}	6.11±0.22^{a}	6.47±0.35^{a}
MCV (fL)	83.17±2.67^{a}	85.03±2.26^{a}	78.55±0.57^{a}	84.69±1.57^{a}	83.29±2.52^{a}
MCH (pg)	27.23±1.04^{a}	27.82±0.09^{a}	25.31±2.38^{a}	27.72±0.31^{a}	27.34±1.29^{a}
MCHC (%)	32.74±0.52^{a}	32.77±0.92^{a}	32.18±2.83^{a}	32.74±0.24^{a}	32.79±0.55^{a}

PCV, Packed cell value; Hb, haemoglobin; RBC, red blood cell; WBC, while blood cell; MCV, mean corpular volume; MCH, mean corpular hemoglobin; MCHC, mean corpular haemoglobin concentration.*Data represents mean ± (SE) values of parameters estimated. Values in the same rows with the same alphabet superscript are not significantly different (p>0.05).

DISCUSSION

Proximate examination of roselle and ginger based diets revealed significant difference (p<0.05) in proximate composition (Table 2). Higher values for crude protein, ash, dry matter and fat were found in ginger based diets followed by roselle based diets and the control. Kumar et al. (2014) reported 41.14 to 42.32 4% crude protein for ginger based diets.

Medicinal plants have been reported to be growth promoters and immune boosters in livestock and fish nutrition (Levic et al., 2008; Kumar et al., 2014; Reverter et al., 2014; Iheanacho et al., 2017a). The findings of the present study on growth performance of *C. gariepinus* juvenile fed roselle and ginger supplemented diets revealed that there were increases in treated groups as compared to the control, although, insignificant (p>0.05) (Table 3). Highest values in terms of final weight (67.51±0.44) and weight gain (31.82±0.63) were observed in fish group fed 4.0 g ginger supplemented diet followed by 4.0 g roselle treated groups. Significant increase in terms of SGR was seen in 4.0 g ginger treated group as compared to the control. Food conversion ratio (FCR) was observed to be highest in fish

group fed 2.0 g roselle supplemented diet and lowest in 4.0 g ginger treated groups. Kumar et al. (2014) reported enhanced growth in Indian catfish fingerling (*Mystus montanus*) fed ginger supplemented diets when compared with the control diet. Iheanacho et al. (2017a) reported significant increases in weight gain, specific growth rate and final weight when *C. gariepinus* juvenile were exposed to varying concentrations (0.25, 0.50, 0.75 and 1.0 g/35 mgL) of ginger as compared to the control. Adewole (2014) reported significant increases ($p<0.05$) in growth parameters (final weight, weight gain, specific rate and relative growth rate) in *C. gariepinus* fed roselle supplemented diets when compared with the control. The positive response to growth in treated fish especially at 4.0 g inclusion level of ginger could be attributed to the high proximate content of ginger (Table 2). Ginger is a good source of protein, mineral elements, vitamins and contains good number of phytochemical constituent that enhance growth and health of animals.

Hematological assays might give a useful guide on the physiological condition of fish (Haghighi and Rohani, 2013). The present study revealed significant changes in hematological parameters in the study fish. Highest values for PCV, Hb and RBC were seen in fish fed 4.0 g ginger supplemented diet as compared to other treatments and the control. There were no significant changes ($p>0.05$) in MCV, MCH and MCHC among treated groups as compared to the control. The present study revealed that ginger and roselle enhanced non specific immune response in *C. gariepinus*. The result of the present study collaborates the findings of Haghighi and Rohani (2013) who reported significant increases in RBC, PCV, Hb and WBC values in rainbow trout (*Oncorhynchus mykiss*) fed ginger supplemented diets when compared to the control. Kumar et al. (2014) also reported significant increases ($p<0.05$) in RBC, Hb, PCV and WBC values in Indian catfish (*Mystus montanus*) fed ginger based diets as compared to the control. Iheanacho et al. (2017a) reported similar findings when *C. gariepinus* were exposed to varying concentrations of ginger bath for 12 weeks.

Yahaya et al. (2012) opined that medicinal plants (roselle, ginger, ugwu and moringa) significantly increased haematological parameters in albino rat as compared to the control.

Conclusion

The present study revealed that both roselle and ginger supported the growth and hematology of *C. gariepinus* juvenile especially, 4.0 g ginger supplemented diets. The use of costly feed supplements should be discouraged. Since ginger and roselle are readily available all year round, there is need to encourage the local fish farmers to imbibe this feed biotechnology in order to minimize cost of fish production.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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REFERENCES

Abu-Tarboush HM, Ahmed SAB, Alkahtani HA (1997). Some nutritional properties of karkade (*Hibiscus sabdariffa*) seed products. Cereal Chem. 74:352-355

Adewole AM (2014). Effects of roselle as dietary additive on growth and production economy of *Clarias gariepinus*. J. Emerg. Trends Eng. Appl. Sci. 5(7):1-8.jeteas.scholarlinkresearch.com

Alector VA, Osho IB (2009). Formulation of medicinal plants for animal production. invited paper II. Proceedings of the Humboldt Kellog/5th Annual Agric. Conference on formulation of medicinal plants for crop and animal production in Nigeria: Realities and Challenges. 21st – 23rd April, 2009. Federal University of Technology Akure. pp. 7-18.

Dacie JV, Lewis SM (2011). Practical Hematology. 11th edition, New York: ChurchillLivingstone,

Estrella MCP, Mantaring JBV, David GZ (2000). A double blind randomized, controlled trial on the use of malunggay (Moringa oleifera) for augmentation of the volume of the breast milk among nonnursing mothers of preterm infants. Philippine J. Ped. 49:306.

Gomiero LM, Braga FMS (2005). The condition factor of fishes from two river basins in Sao Paulo state, Southeast of Brazil. Acta Sci. 27:73-78.

Haghighi M, Mostafa SR (2013). The effects of powdered ginger (*Zingiber officinale)* on the haematological and immunological parameters of rainbow trout *Oncorhynchus mykiss.* J. Med. Plant Herb. Ther. Res. 1:8-12.

Iheanacho S, Ogunji JO, Ogueji EO, Nwuba LA, Nnatuanya IO, Ochang SN, Mbah CE, Ibrahim BU, Haruna M (2017a). Comparative Assessment of Ampicillin Antibiotic and Ginger (*Zingiber officinale)* effects on Growth, Haematology and Biochemical Enzymes Of *Clarias gariepinus* Juvenile. J. Pharmacog. Phytochem. 6(3):761-767.

Iheanacho SC, Nworu SA, Ogueji EO, Nnatuanya I, Mbah CE, Anosike F, Okoye C, Ibrahim UB, Kogi E, Haruna M (2017b). Comparative assessment of proximate content and Organoleptic quality of African catfish (*Clarias gariepinus*) processed by smoking and solar drying methods. Afr. J. Agric. Res. 12(3):2824-2829.

Iheanacho S, Ikwo T, Igweze N, Chukwuidha C, Ogueji EO, Onyeneke R (2018). Effect of different dietary inclusion levels of melon seed (*Citrullus lanatus*) peel on growth, Haematology and Histology of *Oroechromis niloticus* Juvenile. Turkish J. Fish. Aquat. Sci. 18 (3):377-384.

Ijeomah AU, Ugwuona FU, Abdullah H (2012). Phytochemical composition and antioxidant properties of *Hibiscus sabdariffa* and *Moringa oleifera*, Nigerian J. Agric. Environ. 8(1):10-16.

Kim JS, Park HW, Yang JH, Shin TY, KimYC, Baek N, Kim SH (2008). "Cytotoxic components from the dried rhizomes of *Zingiber officinale* Roscoe". Arch. Pharmacol. Res. 31(4):415-418.

Kumar IV, Chettadurai G, Veri T, Peeran SH, Mohanraj J, (2014). Medicinal plants AS immunostimulants for health management in Indian Catfish. J. Coast. Life Med. 2(6):426-430, Doi: 10.12908IJCCM.2.20141432

Levic JG, Sinisia MG, Djuragic O, Slavica S (2008). Herbs, organic acids as alternative to antibioticgrowth promoter. Arch. Zootechn. 11:15-1

Okeke IN, Sosa A (2003). Antibotic resistance in Africa – discerning the

enemy as plotting a defence. Features Afr. Health 25(3):10-15.
Pietta PG (2000). Flavonoids as antioxidants. J. Nat. Prod. 63(7):1035-1042.
Reverter M, Bontemps N, Lecchini D, Banaigs B, Sasal P (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current statue and future perspectives. Aquaculture 433:50-61.
Rico A, Phu TM, Satapornvanit K, Min J, Shahabuddin AM, Henrik PJG, Murray FJ, Little DC, Dalsgaard A, Van den Brink PJ (2013). Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. Aquaculture 412:231-243.
Romero J, Carmen GF, Paola N (2012). Antibiotics in Aquaculture – Use, Abuse and Alternatives. In Health and environment in aquaculture. InTech. In: Tech, Available from: http://www.intechopen.com/books/health-and-environment-in-aquaculture/antibioticsin-aquaculture-use-abuse-and-alternatives.
Shubha RS (2015). Medicinal uses of ginger (*Zingiber officinale* Roscoe) improves growth and enhances immunity in aquaculture. Int. J. Chem. Stud. 3(2):83-87.
US Food and Drug Administration (USDA) (2014). Code of Federal Regulations,; Essential oilsoleoresins (solvent-free), and natural extractives (including distillates): ''Substance generally Recognised As Safe'' Title 21, part 182, sec. 182.20.
Visek WJ (1978). The mode of growth promotion by antibiotics. J. Anim. Sci. 46:1447-1469.
World Bank (2006). Aquaculture: Changing the Face of the Waters. Meeting the Promies and Challenges of Sustainable Aquaculture. The World Bank, Wsahington.
Yahaya T, Okpuzor J, Ajayi T (2012). The prophylactic efficacy of Roselle (*H. Sabdariffa),* Moringa (*Moringa oleifera),* Ginger (*Zingiber officinale)* and Ugwu (*T. Occidentalis)* on the hematology and serum protein of albino rats (*Rattus norvegicus)* exposed to cement dust. J. Med. Plant 6:189-196.

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Full Length Research Paper

Diversity and extracellular enzymes of endophytic fungi associated with *Cymbidium aloifolium* L.

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Cymbidium aloifolium **is an epiphytic orchid used in treatment of many human ailments. The endophytic fungi associated with orchids are diverse and have potential to produce many bioactive compounds including extracellular enzymes. A total of 165 endophytic fungi representing 22 different fungal species were obtained from root, leaf and flowers of *C. aloifolium*. The colonization rate (CR) and isolation rate (IR) varied with different plant parts and was highest in root (CR = 40.6%, IR = 0.83) followed by leaf (CR = 32.12%, IR = 0.66) and flower (CR = 27.27%, IR = 0.56). The diversity of isolated endophytic fungi in root, leaf and flower was determined; Shannon-Wiener index (H') was highest in root (H' = 2.64) followed by leaf (H' = 2.12) and flower (H' = 1.5). Simpson diversity index (D') was high in root (D' = 0.93) with a maximum of 16 species, followed by leaf (D'= 0.88) with 9 species and flower (D' = 0.78) with 5 species. Shannon evenness index (J') was highest in leaf (J' = 0.96) followed by root (J'= 0.95) and flower (J' = 0.93). The endophytic fungi subjected for production of extracellular enzymes; 93% produced phosphatase, 80% cellulase, 70% amylase, 63.33% protease, 30% pectinase, 23.33% lipase and 10% laccase.**

Key words: *Cymbidium aloifolium*, orchid, endophytic fungi, diversity, extracellular enzymes.

INTRODUCTION

Cymbidium aloifolium L. is an herbaceous epiphytic orchid growing on tree trunks at an altitude of 300 to 2900 m (Rajbhandari and Dahal, 2004; Pothangbam and Nirmala, 2011). It is used for ornamental purposes (Subedi and Paudyal, 2001) and also as medicine to cure ear ache, cuts, wounds, paralysis, boils, fever and for joining the fractured bones (Mukul et al., 2007). The endophytic fungi are the important components of epiphytic orchids which exhibited gamut of fungal dependency for nutrition; the fungi in turn help the orchid to assimilate nutrition from the bark of trees by the production of extracellular enzymes (Benzing and Friedman, 1981).

The study of Orchidaceae family is interesting as its entire species (approx. 25,000 sp.) are heterotrophic and nourished by endophytic fungi during the early stages of

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development (Smith and Read, 1997). The fungal colonization occurs in roots, leaves or other structures and process depends on the genera of the fungus and the orchid species (Rasmussen and Whigham, 2002). Mycorrhizal fungi in orchid promote the germination of seeds, stimulate the development and growth of protocorms, seedlings, adult plants and tubers (Dearnaley, 2007; Takahashi et al., 2007). The orchid and fungus exhibit mutuality, with orchid providing vitamins and sugars to the fungus, while fungus transfers water, mineral salts and up to 85% of orchid's carbon requirement (Pridgeon et al., 2001); hence, the association between endophytic fungi and orchid is symbiotic. The endophytic fungi have been explored for diverse range of valuable compounds like enzymes and secondary metabolites. The production of bioactive compounds in fungi depends on its host habitat (Hyde and Soytong, 2008).

The extracellular enzymes produced by fungi are gaining importance in textile, leather, confectionery, beverage, food industry, agriculture and human health due to its stability at various extreme conditions such as high temperature and pH (Benjamin and Pandey, 1998). The amylases convert starch into sugar syrup and used it in food and pharmaceutical industry. The lignocelluloses abundantly found in nature are degraded by cellulase and hence play an important role in the cycling of carbon and other nutrients. The cellulases along with hemicellulase and pectinase are used in degradation of lignocellulosic materials that serve as raw material for fermentation and paper industries (Dyk and Pletschke, 2012). The protease provides softness to the silk fiber; it is also used in hide dehairing, as contact lens cleaners, healing and management of skin ulcers by removal of necrotic materials. The pectinase is an important enzyme required in food and wine industry. The laccases are used in pulp and paper industry; also it has environmental applications such as bioremediation. The lipase has vast industrial application in detergent, oil, fat, dairy and therapeutic industries (Benjamin and Pandey, 1998). The microbes with phosphate solubilizing ability enhance the availability of soluble phosphate and thus promote the plant growth (Ponmurugan and Gopi, 2006).

The endophytic fungi produce several enzymes to support its host growth and defense against microbial pathogens (Sunitha et al., 2012). The endophytic fungi invade the plant tissues by producing extracellular hydrolases like pectinase, cellulase, lipase, laccase, etc. (Ward, 2012). These extracellular enzymes degrade lignocellulosic fibers; the hydrolytic enzymes like xylanases and cellulases degrade polysaccharides and oxidative lignolytic system produces laccases, ligninases and peroxidases which degrade lignin and opens the phenyl ring system. The enzyme production and regulation by the endophytic fungi is due to its genetic recombination with the host which has evolved with evolutionary time (Priest, 1984). However, the enzymes produced by endophytic fungi have been barely exploited for industrial interest (Correa et al., 2014).

The endophytic fungi associated with this epiphytic orchid have remained unexplored vicinity for the production of extracellular enzymes and thus may prove to be a possible source in obtaining enzymes with different potentialities (Schulz et al., 2002). Hence, in the present study, an attempt was made to isolate endophytic fungi from different parts of *C. aloifolium* like root, leaf and flower; diversity of endophytic fungi was studied. These endophytic fungal isolates were further evaluated for the production of industrially important extracellular enzymes.

MATERIALS AND METHODS

Endophytic fungi isolation and identification

The *C. alofolium* was collected during the flowering period of March to June, 2015, from different regions of Western Ghats of Karnataka such as Sringeri, Chikkamagaluru, Kemmanagundi and Shivamoga. The samples were collected and brought to laboratory, washed with tap water, cut into 0.5 cm^2 segments and surface sterilized with 95% ethanol (1 min), 4% NaOCl (5 min) and rinsed with sterile distilled water 5 times. The sterilized segments were teased using sterile blade and placed aseptically on potato dextrose agar (PDA) plate amended with 50 µg/ml tetracycline. The plates were incubated in dark conditions at 30°C and observed daily for up to four weeks for the fungal colonies. The endophytic fungi were isolated from root, leaf and flower (80 segments each) of *C. aloifolium* using a modified procedure of Zhu et al. (2008). The pure cultures of endophytic fungi were transferred to PDA slants and then stored at 4°C. The identification of fungi was done by morphological methods (Ellis, 1971; Sutton, 1980; Barnett and Hunter, 1998).

Data analysis

The frequency of endophytic fungi colonizing different organs of *C. aloifolium* such as root, leaf and flower was determined. The absolute frequency (f) was estimated as the number of each endophytic fungi isolated from each plant organ (Larran et al., 2002). The relative frequencies (fr) of isolation was calculated for each organ; the number of isolates of each endophytic fungal species was divided by the total number of isolates obtained in each organ and expressed as percentage. The isolation rate (IR) was determined by dividing the number of endophytic fungal isolates from an organ divided by the total number of tissue segments. The percentage of colonization rate (CR) was determined by dividing the total number of endophytic fungal isolates obtained from each organ segments divided by the total number of isolates obtained from overall organ segments incubated multiply by 100. The Simpson's diversity index (D'), Shannon-Wiener index (H'), evenness (J) and species richness (S) were determined as described by Magurran (1988).

Detection of extracellular enzyme production in endophytic fungi

The ability of the endophytic fungi to produce extracellular enzymes: amylase, lipase, pectinase, cellulase, protease, laccase and phosphatase, were screened using different solid media

(Hankin and Anannostakis, 1975; Neha et al., 2015). The production of extracellular enzymes was assessed by growing each of the endophytic fungi on PDA for 1 week and 5 mm of these mycelia plugs were placed on the solid media having different substrates for respective enzymes. After incubation for 5 to 7 days at room temperature, the diameter of hydrolysis and fungal colony were measured; enzyme index was calculated (Florencio et al., 2012).

Enzyme index = Diameter of hydrolysis / Diameter of fungal colony.

Amylase

Amylase production was assessed by growing endophytic fungi on glucose yeast extract peptone agar (GYP: glucose, 1 g; yeast extract, 0.1 g; peptone, 0.5 g; agar, 15 g, distilled water, 1 L) with 0.2% soluble starch; pH 6.0. After incubation, plates were flooded with 1% iodine in 2% potassium iodide. The zone of clearance around the blue background indicated the production of amylase.

Lipase

The lipase production was assessed by growing endophytic fungi on peptone agar (peptone, 10 g; NaCl 5 g; $CaCl_2.2H_2O$ 0.1 g; agar, 15 g; distilled water 1 L; pH 6.0) with 1% Tween 20 (sterilized and added separately to the media before pouring). The visible precipitate around the colony indicated the presence of lipase. The precipitate formed was due to the formation of calcium salts of lauric acid liberated by lipase.

Pectinase

The pectinase production was assessed by growing fungi on pectin agar medium (pectin 5 g; yeast extract 1 g; agar, 15 g; distilled water, 1 L, pH 5.0). After incubation, the plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium bromide. A clear zone around the colony showed the production of pectinase.

Cellulase

The cellulase production was detected by growing fungi on glucose yeast extract peptone agar with 0.5% carboxy-methyl cellulose. After incubation, the plates were flooded with 0.2% aqueous congo red solution and destained with 1 M NaCl for 15 min. The yellow zone around the colony showed the presence of cellulase.

Protease

Glucose yeast peptone agar with gelatin at pH 6.0, was used to detect the production of protease by endophytic fungi. Gelatin (8%) in water was sterilized separately and added to GYP medium at the rate of 5 ml per 100 ml. The clear zone around the colonies showed the presence of protease which was enhanced when the plates were flooded with saturated ammonium sulphate.

Laccase

Glucose yeast peptone agar with 0.05 g of 1 napthol L^{-1}, pH 6, was used to detect the production of laccase. The laccase production was indicated by change from colourless media to blue colour due to the oxidation of 1-napthol.

Phosphatase

The phosphate solubilizing ability of the fungal isolates were tested using the modified procedure of Neha et al. (2015) on Pikovskaya's solid agar media (glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_20$, 0.1 g; KCl, 0.2 g; yeast extract 0.5 g; $MnSO_4.H_2O$, 0.002 g, $FeSO_4.7H_2O$ 0.002 g and Agar 15 g, distilled water 1 L) with bromo phenol blue at a concentration of 0.003%. After 3 to 5 days of incubation, clear zone around the fungal colony indicated the ability of fungus to solubilize inorganic phosphorous.

Statistical analysis

All the experiments were performed in triplicates and the means of the enzyme index were analyzed statistically and Duncan multiple range test was carried out using SPSS software program version 20 (Duncan, 1955).

RESULTS

Colonization of endophytic fungi in *C. aloifolium*

A total of 165 endophytic fungi representing 22 different species were obtained from 240 organ fragments analyzed; 80 fragments were taken from each of the root, leaf and flower of *C. aloifolium*. Sixty seven endophytic fungi were obtained from root, 53 from leaf and 45 from flower; all the isolates belonged to *Ascomycota* except one isolate which was *Basidiomycota* and belonged to the groups of *Dothideomycetes*, *Eurotiomycetes*, *Sordariomycetes*, *Botryospaeriaceae*, and *Leotiomycetes*. The root was more frequently associated with *Aspergillus terreus* followed by *Colletotrichum gleosporidioides* and other fungi*;* leaf was more frequently associated with *Penicillium chrysogenum* followed by *Alternaria alternata, Trichoderma* species; in flower, *Fusarium oxysporum* was most frequently isolated followed by *Cyperus rotundus*, *Penicillium purpurogenum* and others. The endophytic fungi were found to be associated with all the parts of *C. aloifolium* analyzed and the extent of colonization varied with different organs; colonization rate and isolation rate was highest in root (CR = 40.6%, IR=0.83) followed by leaf (CR = 32.12%, IR=0.66) and flower (CR= 27.27%, IR=0.56) (Table 1). Shannon diversity index (H') was highest in the root (H'=2.64) followed by leaf (H'=2.12) and lowest in flower (H'=1.5). Simpson diversity index (D') was high in root (D'= 0.93) with a maximum of 16 different species, followed by leaf (D'=0.88) with 9 species and flower (D'=0.78) with 5 species. Shannon evenness index (J') was highest in leaf (J'=0.96) followed by root (J'=0.95) and flower (J'=0.93) (Table 2).

Screening of endophytic fungi for extracellular enzyme production

The 30 different endophytic fungi obtained from root, leaf

Table 1. Frequency of Endophytic fungi isolated from different parts of *C. aloifolium*.

S/N	Endophytic fungi	Roots		Leaf		Flower		Total	
		f	fr (%)	f	fr (%)	f	fr (%)	f	fr(%)
1	*Aspergillus japonicas*	3	4.47	0	0	0	0	3	1.81
2	*Curvularia lunata*	3	4.47	3	5.66	0	0	6	3.63
3	*Nigrospora* spp.	1	1.5	0	0	0	0	1	0.60
4	*Colletotrichum gleosporidioides*	8	11.94	0	0	0	0	8	4.84
5	*Rhizoctonia* spp.	4	5.97	7	13.20	0	0	11	6.66
6	*Xylaria* spp.	3	4.47	0	0	0	0	3	1.81
7	*Trichoderma* spp.	5	7.46	8	15.09	0	0	13	7.87
8	*Fusarium chlamydosporum*	3	4.47	0	0	0	0	3	1.81
9	*Penicillum citrinum*	4	5.97	4	7.54	0	0	8	4.84
10	*Helminthosporium* spp.	2	2.98	0	0	0	0	2	1.21
11	*Curvularia* spp.	3	4.47	0	0	0	0	3	1.81
12	*Aspergillus sydowii*	7	10.44	4	7.54	0	0	11	6.66
13	*Cladosporium* sp.	4	5.97	0	0	8	17.78	12	7.27
14	*Aspergillus terreus*	10	14.92	0	0	0	0	10	6.06
15	*Alternaria alternata*	3	4.47	8	15.09	0	0	11	6.66
16	*Fusarium oxysporum*	4	5.97	0	0	15	33.33	19	11.51
17	*Penicillium chrysogenum*	0	0	10	18.86	0	0	10	6.06
18	*Colletotrichum truncatum*	0	0	4	7.54	0	0	4	2.42
19	*Bipolaris* spp.	0	0	5	9.43	0	0	5	3.03
20	*Taloromyces rotundus*	0	0	0	0	10	22.22	10	6.06
21	*Penicillium purpurogenum*	0	0	0	0	9	20	9	5.45
22	*Cylindrocephalum* spp.	0	0	0	0	3	6.66	3	1.81
Total		67	100	53	100	45	100	165	100
Colonization rate (CR%)		40.6		32.12		27.27		100	
Isolation rate (IR)		0.83		0.66		0.56		0.68	

F, Absolute frequency; fr, relative frequency.

Table 2. Diversity, evenness and species richness of endophytic fungi isolated from different organs of *C. aloifolium*.

C. aloifolium part	H'	D'	J'	S
Root	2.64	0.93	0.95	16
Leaf	2.12	0.88	0.96	9
Flower	1.5	0.78	0.93	5

Shannon-Wiener (H'), Simpson (D') diversity indices, evenness (J') and richness (S).

and flower of *C. aloifolium* were screened for the production of extracellular enzymes: amylase, lipase, laccase, protease, pectinase, cellulase, and phosphatase (Table 3). The endophytic fungi producing extracellular enzymes were assessed based on the formation of halo zone around the fungal colony and enzyme index were calculated (Figure 1). The endophytic fungi produced one or the other extracellular enzyme necessary for penetrating and colonizing their host. In the present study, none of the endophytic fungi were able to produce all the seven enzymes screened but, all the fungi produced more than one enzyme, except *Curvularia* species (CAR11) and *A. alternata* (CAL8) which produced only cellulase enzyme (Figure 2). Ninety nine percent of endophytic fungi produced phosphatase followed by 80% cellulase, 70% amylase, 63.33% protease, 30% pectinase, 23.33% lipase and 10% laccase (Figure 3).

Production of phosphatase enzyme

Most of the endophytic fungi (93.33%) produced phosphatase enzyme; enzyme index for phosphatase ranged from the highest of 1.58 in *Colletotrichum truncatum* (CAL7) to lowest of 1.04 in *Helminthosporium* species (CAR10) indicating the phosphatase produced was almost similar in all the endophytic fungi screened. *A. alternata* (CAL8) and *Curvularia* spp. (CAR11) from root were negative for phosphatase.

Production of cellulase enzyme

Eighty percent of endophytic fungi produced cellulase

Table 3. Extracellular enzyme production by endophytic fungi isolated from *C. aloifolium*.

S/N	Fungal isolate		Endophytic fungi	Enzyme index						
				Amylase	Lipase	Laccase	Protease	Pectinase	Cellulase	Phosphatase
1	CAR1	Root	*Aspergillus japonicas*	2.15±0.11^{hi}	0.00^{a}	0.00^{a}	3.68±0.13^{h}	1.52±0.06^{d}	2.28±0.32^{hi}	1.41±0.15^{k}
2	CAR2		*Curvularia lunata*	2.21±0.16^{hi}	0.00^{a}	0.00^{a}	2.43±0.49^{fg}	0.00^{a}	1.45±0.16^{def}	1.1±0.02^{bcd}
3	CAR3		*Nigrospora* spp.	2.15±0.1^{hi}	3.46±0.14^{g}	1.41±0.18^{b}	0.00^{a}	0.00^{a}	2.5±0.14^{i}	1.26±0.03^{fghi}
4	CAR4		*C. gleosporidioides*	3.07±0.08^{j}	0.00^{a}	0.00^{a}	5.3±0.34^{j}	1.85±0.04^{e}	7.99±0.16^{m}	1.37±0.05^{ijk}
5	CAR5		*Trichoderma* sp.	2.15±0.11^{hi}	0.00^{a}	0.00^{a}	4.56±0.38^{i}	1.41±0.04^{cd}	4.5±0.29^{l}	1.24±0.05^{efgh}
6	CAR6		*Xylaria* spp.	1.88±0.15^{g}	1.93±0.3^{c}	1.58±0.08^{c}	2.26±0.1^{f}	0.00^{a}	1.33±0.11^{bcde}	1.26±0.13^{fghi}
7	CAR7		*Rhizoctonia* spp.	0.00^{a}	0.00^{a}	0.00^{a}	1.73±0.28^{de}	0.00^{a}	1.57±0.15^{ef}	1.07±0.05^{bc}
8	CAR8		*Fusarium chlamydosporum*	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.49±0.35^{ef}	1.33±0.0^{ghijk}
9	CAR9		*Penicillium citrinum*	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.51±0.12^{d}	1.36±0.06^{cde}	1.19±0.01^{cdef}
10	CAR10		*Helminthosporium* spp.	0.00^{a}	0.00^{a}	0.00^{a}	2.42±0.04^{fg}	0.00^{a}	0.00^{a}	1.04±0.01^{b}
11	CAR11		*Curvularia* spp.	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.64±0.03^{f}	0.00^{a}
12	CAR12		*Aspergillus sydowii*	0.00^{a}	0.00^{a}	0.00^{a}	1.73±0.28^{de}	1.41±0.11^{cd}	0.00^{a}	1.28±0.07^{fghij}
13	CAR13		*Cladosporium* spp.	2.32±0.07^{i}	0.00^{a}	0.00^{a}	2.61±0.5^{g}	0.00^{a}	1.91±0.07^{g}	1.12±0.01^{bcde}
14	CAR14		*Aspergillus terreus*	1.21±0.02^{b}	0.00^{a}	0.00^{a}	0.00^{a}	1.24±0.09^{b}	1.15±0.01^{bc}	1.24±0.04^{efgh}
15	CAR15		*Alternaria* alternata	1.7±0.39^{f}	0.00^{a}	0.00^{a}	1.93±0.2^{e}	0.00^{a}	1.17±0.1^{bc}	1.16±0.02^{bcdef}
16	CAR16		*Fusarium oxysporum*	1.25±0.02^{b}	2.67±0.17^{e}	0.00^{a}	2.23±0.15^{f}	0.00^{a}	0.00^{a}	1.13±0.02^{bcde}
17	CAL1	Leaf	*Penicillium chrysogenum*	2.15±0.05^{hi}	0.00^{a}	0.00^{a}	3.68±0.13^{h}	2.02±0.13^{f}	2.42±0.14^{i}	1.42±0.03^{k}
18	CAL2		*Aspergillus sydowii*	1.35±0.02^{bc}	3.18±0.14^{f}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.24±0.01^{efgh}
19	CAL3		*Trichoderma* spp.	1.37±0.17^{bcd}	0.00^{a}	0.00^{a}	1.73±0.28^{de}	0.00^{a}	1.18±0.04^{bc}	1.22±0.08^{defg}
20	CAL4		*Rhizoctonia* spp.	1.58±0.08^{ef}	0.00^{a}	0.00^{a}	1.58±0.27^{cd}	0.00^{a}	1.18±0.04^{bc}	1.36±0.03^{hijk}
21	CAL5		*Curvularia lunata*	2.26±0.13^{i}	0.00^{a}	0.00^{a}	1.46±0.14^{bcd}	0.00^{a}	1.09±0.04^{b}	1.22±0.1^{efg}
22	CAL6		*Penicillium citrinum*	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.19±0.05^{bcd}	1.54±0.13^{lm}
23	CAL7		*Colletotrichum* truncatum	1.45±0.02^{cde}	0.00^{a}	0.00^{a}	1.46±0.14^{bcd}	1.23±0.14^{b}	2.28±0.32^{hi}	1.58±0.1^{m}
24	CAL8		*Alternaria alternata*	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.39±0.16^{cdef}	0.00^{a}
25	CAL9		*Bipolaris* spp.	1.65±0.04^{f}	1.98±0.22^{c}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.39±0.09^{jk}
26	CAF1	Flower	*Fusarium oxysporum*	1.55±0.03^{def}	0.00^{a}	0.00^{a}	0.00^{a}	1.31±0.24^{bc}	3.6±0.28^{k}	1.57±0.02^{m}
27	CAF2		*Talaromyces rotundus*	1.45±0.02^{cde}	2.25±0.16^{d}	0.00^{a}	1.66±0.05^{de}	0.00^{a}	2.36±0.16^{i}	1.36±0.01^{hijk}
28	CAF3		*Penicillium purpurogenum*	0.00^{a}	0.00^{a}	1.79±0.09^{d}	1.32±0.03^{bc}	0.00^{a}	2.79±0.14^{j}	1.46±0.03^{k}
29	CAF4		*Cladosporium* spp.	2.04±0.03^{gh}	0.00^{a}	0.00^{a}	1.16±0.02^{b}	0.00^{a}	0.00^{a}	1.17±0.08^{cdef}
30	CAF5		*Cylindrocephalum* spp.	2.26±0.13^{i}	1.61±0.2^{b}	0.00^{a}	0.00^{a}	0.00^{a}	2.05±0.1$^{gh=}$	1.22±0.01^{defg}

The values represent mean of enzyme index ± SD, n=3; Mean values followed by the same letter are not significantly different according to DMRT at $p< 0.05$.

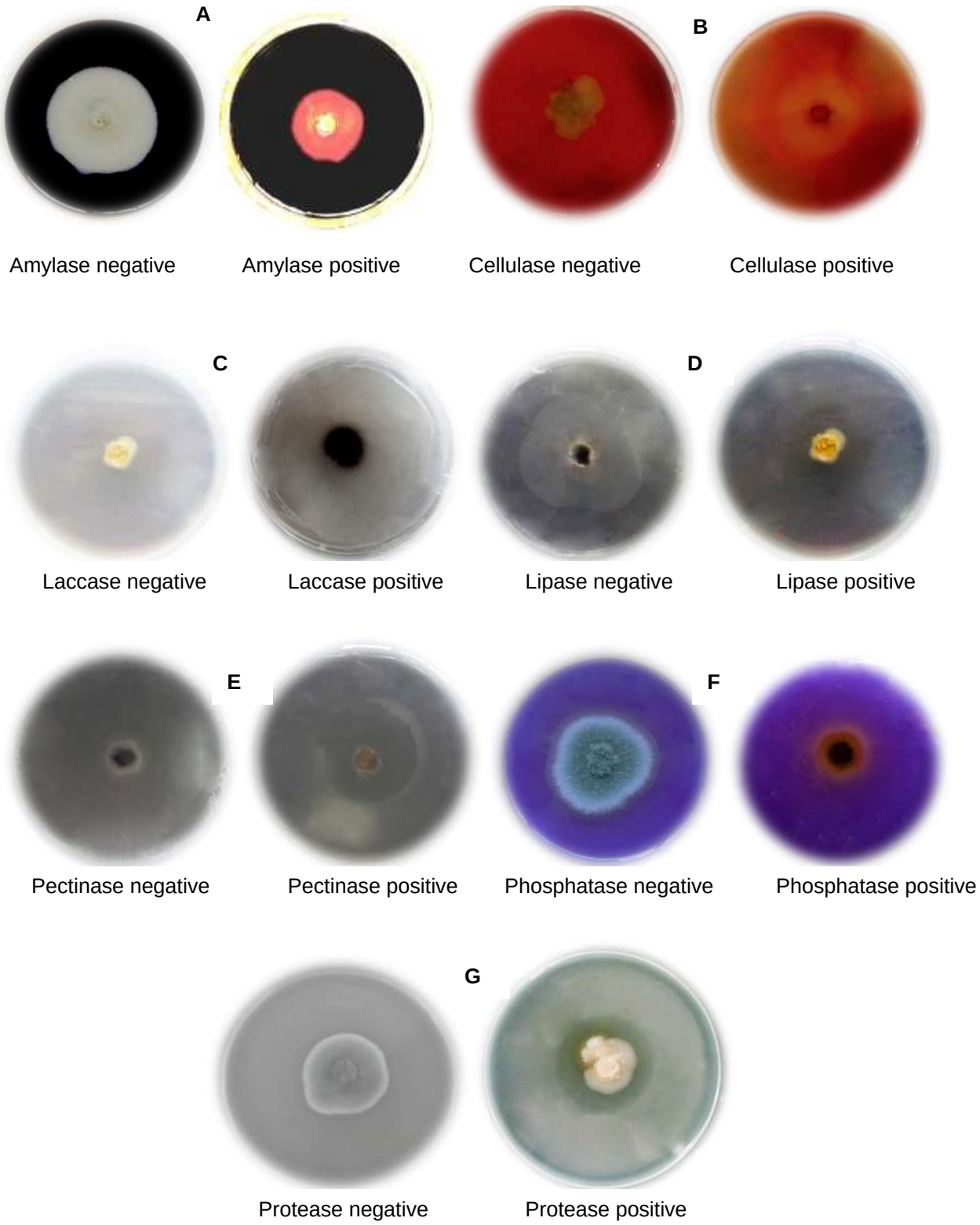

Figure 1. Production of extracellular enzymes by endophytic fungi isolated from *C. aloifolium*.

enzyme and their enzyme index varied with different fungi; the highest enzyme index was 7.99 by *Colletotrichum gloeosporioides* (CAR 4), followed by 4.5 by *Trichoderma* spp. (CAR5), 3.6 by *F. oxysorum* (CAF

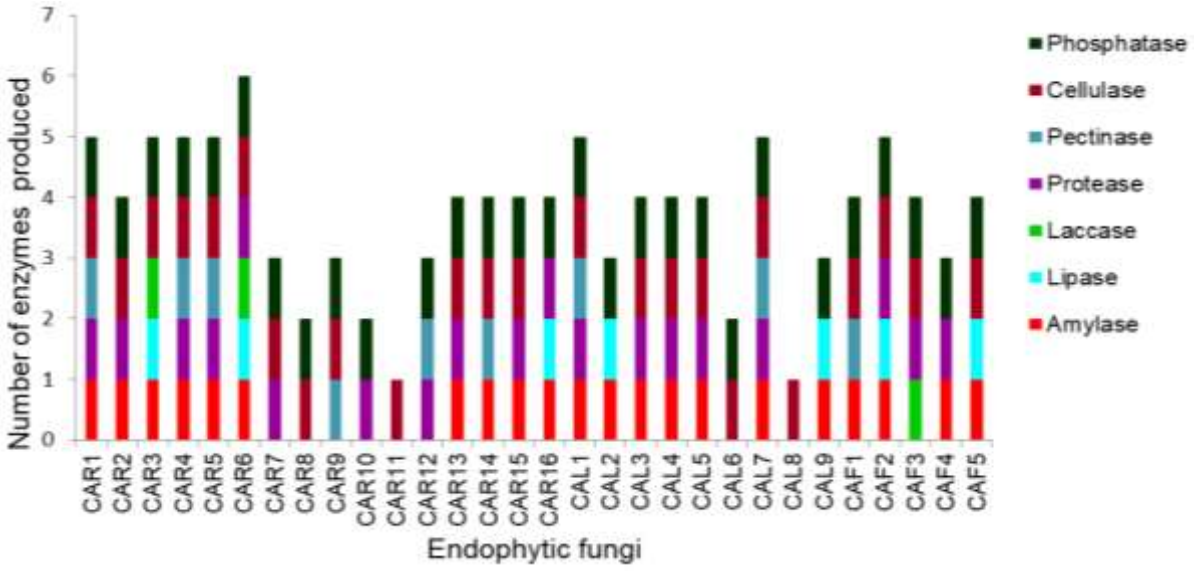

Figure 2. Production of extracellular enzymes by endophytic fungi of *C. aloifolium*.

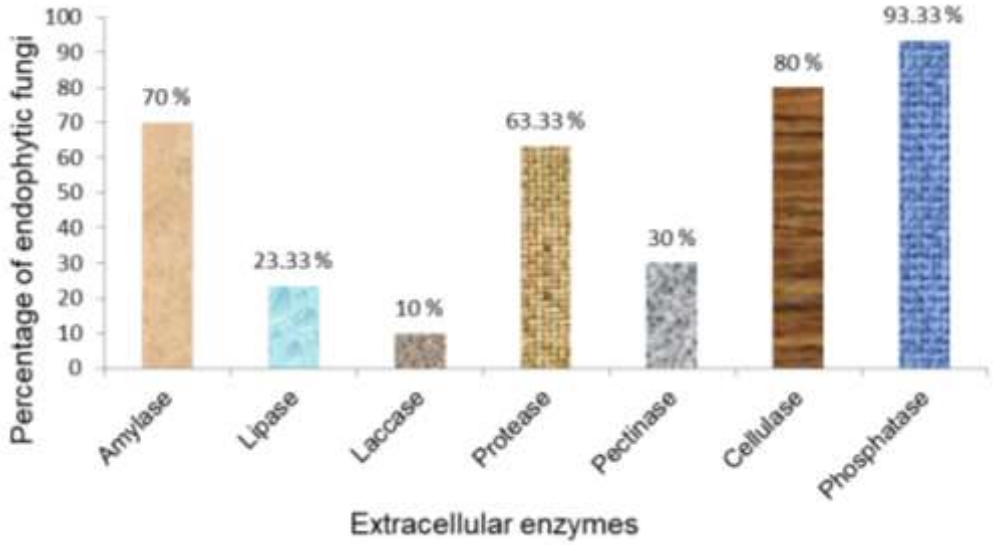

Figure 3. Percentage of endophytic fungi of *C. aloifolium* producing extracellular enzymes.

1) and lowest enzyme index was 1.09 by *Curvularia lunata* (CAL5). The cellulase enzyme was produced by all the fungi screened except CAR10, CAR12, CAR16, CAL2, CAL9, and CAF4 which showed cellulase negative results.

Production of amylase enzyme

The 70% of endophytic fungi produced amylase enzyme and enzyme index ranged from the highest which was 3.07 by *C. gloeosporioides* (CAR 4) to the lowest of 1.21 by *A. terreus* (CAR14). The enzyme index varied among the fungi producing amylase. The amylase was produced by all the endophytic fungi except CAR7, CAR8, CAR9, CAR10, CAR11, CAR12, CAL6, CAL8, and CAF3 which were negative for amylase.

Production of protease enzyme

The 63.33% of endophytic fungi produced protease enzyme. The highest protease enzyme was produced by *C. gloeosporioides* (CAR4) with enzyme index of 5.3,

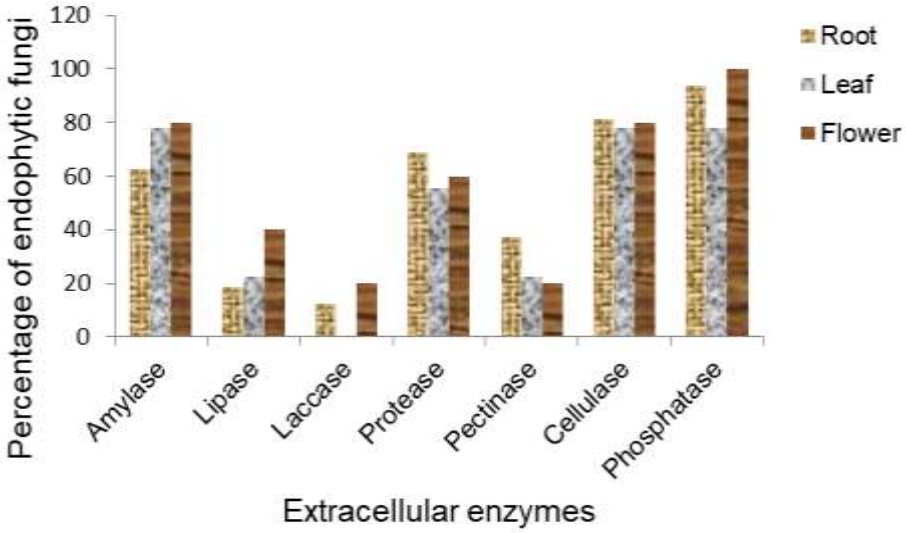

Figure 4. Percentage of endophytic fungi associated with root, leaf and flower of *C.aloifolium* producing extracellular enzymes.

followed by *Trichoderma* spp. (CAR5) with enzyme index of 4.56, *A. japonicas* (CAR 1) with enzyme index of 3.68 and all the other fungi produced protease with an enzyme index which ranged from 2.43 to 1.16; lowest enzyme index was in *Cladosporium* species (CAF4).

Production of pectinase enzyme

The pectinase enzyme was produced by 30% of endophytic fungi; significant enzyme index was 2.02 by *P. chrysogenum* (CAL1) and least was 1.23 by *C. truncatum* (CAL7). The endophytic fungi producing pectinase enzyme were CAR1, CAR4, CAR5, CAR9, CAR12, CAR14, CAL1, CAL7, and CAF1.

Production of lipase enzyme

The lipase was produced by 23.33% endophytic fungi. The prominent enzyme index was 3.46 by *Nigrospora* species (CAR3) followed by 3.18 in *Aspergillus sydowii* (CAL2); 2.67 by *F. oxysporum* (CAR16), 2.25 by *T. rotundus* (CAF2), 1.93 by *Xylaria* species (CAR6), 1.98 by *Bipolaris* species (CAL9) and 1.61 by *Cylindrocephalum* (CAF5).

Production of laccase enzyme

The laccase was produced only in 10% of the endophytic fungi; three isolates CAF3, CAR6 and CAR3 produced laccase. The significant amount was produced by CAF3 (*P. purpurogenum*) with the enzyme index of 1.79 followed by 1.58 by CAR6 and 1.41 by *Nigrospora* spp. (CAR3).

Comparison of the endophytic fungi from different parts of C. aloifolium producing extracellular enzymes

The production of extracellular enzymes varied with the endophytic fungi obtained from different parts of *C. aloifolium*. Eighty percent of endophytic fungi associated with flower, produced amylase followed by leaf endophytic fungi (77.77%) and root endophytic fungi (62.5%). Forty percent of flower endophytic fungi produced lipase followed by leaf endophytic fungi (22.22%) and root (18.75%). Twenty percent of flower endophytic fungi produced laccase followed by root endophytic fungi (12.5%) but there was no laccase production in the endophytic fungi from leaves. The 68.75% of root endophytic fungi produced protease followed by flower endophytic fungi (60%) and leaf endophytic fungi (55.55%). The root endophytic fungi were significant pectinase producers with 37.5% fungi producing it which was followed by leaf endophytic fungi (22.22%) and flower endophytic fungi (20%). The 81.25% of root endophytic fungi produced cellulase followed by flower endophytic fungi (80%) and leaf endophytic fungi (77.77%). All the endophytic fungi associated with flower produced phosphatase enzyme followed by root endophytic fungi (93.75%) and leaf (77.77%) (Figure 4).

DISCUSSION

C. aloifolium is a potent medicinal epiphytic orchid in the Indian system of medicine, traditionally used in treatment of many human ailments (Radhika et al., 2013). The endophytic fungi associated with this orchid have vital role in its life cycle. In the present study, 165 endophytic

fungi were obtained from different parts of *C. aloifolium*; root represented the maximum number of isolates followed by leaf and stem. All the fungal isolates belonged to *Ascomycota* except one which was *Basidiomycota* and they belonged to the groups of *Dothideomycetes*, *Eurotiomycetes*, *Sordariomycetes*, *Botryospaeriaceae* and *Leotiomycetes*. Selosse et al. (2004) have also found *Ascomycetes* to be predominating than *Basidiomycete* fungi in roots of *Epipactis microphylla* (Ehrh.). The Bidartondo et al. (2004) have designated *Ascomycetes* as the potential mycorrhizal fungi in orchids. Our findings are in accordance with those of Stone et al. (2004) who have reported that the endophytic fungi of non-grass hosts represent broader range of taxa mainly from several orders and families of *Ascomycetes* and few from *Basidiomycetes* families. The fungi possessing ascospores with thick cell wall and gelatinous sheath or appendages are adapted to launch their spores onto the plants hence are the predominant endophytic representatives, whereas *Zygomycetes* and *Basidiomycetes* are poorly represented in endophytic inventories.

The extent of endophytic fungi colonizing the different organ segments of *C. aloifolium* varied significantly. The colonization rate and isolation rate was maximum in roots (CR = 40.6%, IR=0.83) followed by leaf (CR = 32.12%, IR=0.66) and flower (CR= 27.27%, IR=0.56); root was more frequently colonized with *A. terreus*, *C. gleosporidioides*, *A. sydowii*, *Trichoderma* spp., *Rhizoctonia* spp*., Penicillum citrinum*, *Aspergillus japonicus*, *Curvularia* spp., *Xylaria* spp., *Chlamydosporum* spp., *Curvularia* spp*., A. alternata*, *Helminthosporium* spp., and *Nigrospora* spp. Chen et al. (2013) have found that, endophytic fungi associated with *Dendrobium* spp. were dominated by *Fusarium* spp., *Colletotrichum* species, and *Xylariaceous* fungi. The findings of this study are in agreement with those of Chen et al. (2010) who have reported that significant number of endophytic fungi were isolated from roots when compared with stem and leaves of *Dendrobium loddigessi*; as roots may provide a better niche for endophytic colonization, whereas findings of Uzma et al. (2016) have reported that colonization rates were higher in leaves when compared with roots of medicinal plants. The present results also agrees with those of Bayman et al. (1997) who have reported that the heterogeneity of endophytes in single plants and its organs are greater than between the species.

The diversity indices of the endophytic fungi associated with different *C. aloifolium* parts were analyzed by Shannon diversity index (H') and Simpson diversity index (D') which showed the variations in the endophytic fungal isolates and its species richness within the different organs of this orchid. The highest diversity indices were observed in root followed by leaf and flower; root exhibited maximum species richness with 16 different species followed by leaf with nine different species and flower with five species, whereas Shannon evenness index was highest in leaf followed by root and flower. The species richness signifies the colonization of different endophytic fungal species within a specific organ, whereas evenness index enumerates the identical abundance of the species in a particular tissue. The present research findings are in agreement with Tao et al. (2008) who have reported that different orchid parts like roots, leaves have different endophytic associations and speculated that could be because of the difference in its tissue texture, physiology and chemistry (Barman and Devadas, 2013).

The endophytic fungi obtained from different parts of *C. aloifolium* produced different extracellular enzymes such as, amylase, lipase, laccase, protease, pectinase, cellulase and phosphatase and are promising source of industrially useful enzymes (Zaferanloo et al., 2013). The present study agrees with those of Bezerra et al. (2012) who have reported the richness of endophytic fungi from *Opuntia ficus*-indica Mill and its capacity to produce extracellular enzymes. In the present study, endophytic fungi were able to produce one or the other extracellular enzymes necessary for penetrating and colonizing their host, but none of them produced all the seven enzymes screened. Petrini et al. (1993) have reported that majority of endophytes utilizes a good number of substrates which are likely to be present on the surfaces and in the host cell wall.

The phosphatase enzyme was produced by 93.33% of the endophytic fungi. The *C. truncatum* (CAL7) isolated from leaf produced significant phosphatase followed by *F. oxysporum* (CAF1) isolated from flower. The phosphatase enzyme produced by the endophytic fungi helps to solubilize the available phosphate which can be easily assimilated by the growing plants. Our findings are in accordance with those of Neha et al. (2015) who have reported production of phosphatase in most of the endophytic fungi. The phosphate solubilization could be due to the various organic acids produced by endophytes which in turn results in reduced pH, thus facilitating phosphate solubilization (Illmer and Schineer, 1995).

Eighty percent of endophytic fungi screened produced cellulase enzyme. The enzyme index varied significantly among the endophytic fungi producing cellulase; *C. gloeosporioides* (CAR4) isolated from root exhibited the highest enzyme index for cellulase. The endophytic fungi associated with *C. aloifolium* help its host to assimilate complex carbohydrates like cellulose present in the tree barks by production of cellulase enzyme (Read et al., 2004). The present findings are supported by the findings of Choi et al. (2005) who have reported that endophytic fungi from *Brucea javanica* produced extracellular cellulase which resulted in weight loss of wood blocks; most of the endophytic fungi are degraders of complex carbohydrates such as cellulose available in dead leaves and wood. The highest cellulase activity have also been

reported from several endophytic fungi of Egyptian marine sponge *Latrunculia corticata* (El-Bondkly and El-Gendy, 2012) and also in endophytic *Xylaria* spp. associated with plant tissues (Wei et al., 1996).

The amylase enzyme was produced by 70% of the endophytic fungi screened. The significant amylase was produced by *C. gloeosporioides* (CAR4), isolated from root with an enzyme index of 3.07. The present findings are in accordance with the findings of Amirita et al. (2012), Choi et al. (2005), and Sunitha et al. (2013) who have reported prominent amylase production by endophytic fungi. The amylolytic activity of endophytic fungi helps to degrade starch into simple carbohydrates which can be later assimilated by both fungi and host (Venkatesagowda et al., 2012).

The 63.33% of endophytic fungi produced protease enzyme and *C. gloeosporioides* (CAR4) from root significantly produced protease. The endophytic fungi produce various enzymes including protease which helps them to overcome the host defense barrier and in turn obtain nutrients for their development (Amirita et al., 2012). The present findings agrees with Boyle et al. (2001) and Sunitha et al. (2013) who have reported the production of protease and other enzymes by endophytic fungi which helps in penetrating and colonizing the host plants. Thirty percent of endophytic fungi produced pectinase enzyme which is lesser than those of Sunitha et al. (2013) who reported 62% endophytic fungi of *Alpinia calcarata*, *Bixa orellana* and *Calophyllum inophyllum* producing this enzyme, whereas Choi et al. (2005) have reported that pectinase production was absent in all the endophytic fungi of *Brucea javanica.*

23.33% of the endophytic fungi produced lipase enzyme and *Nigrospora* spp. (CAR3) isolated from root was major lipase producer. The percentage of endophytic fungi that produced lipase in the present study was greater when compared with 3.12% of the endophytic fungi from oil bearing seeds producing lipase, but Sunitha et al. (2013) have reported that 50% of endophytic fungi of *Alpinia calcarata*, *Bixa orellana* and *Calophyllum inophyllum* produced lipase. The laccase was produced by three endophytic fungi, *P. purpurogenum* (CAF3) from flower being the highest producer. The present findings are in concurrence with those of Sajben et al. (2014) who have reported that the endophytic fungi and marine fungi producing laccase are meagre, but Maria et al. (2005) and Uzma et al. (2016) have reported that the endophytic fungi of medicinal plants from Western Ghats failed to produce laccase.

The production of extracellular enzymes varied with the endophytic fungi associated with different organs of *C. aloifolium* like root, leaf and flower. The highest number of endophytic fungi associated with flower produced amylase, lipase, laccase and phosphatase, whereas more number of root endophytic fungi produced protease, pectinase and cellulase enzyme. The present findings are in accordance with those of Alves et al. (2014) who have reported that the predominance of extracellular enzyme depends on the type of samples.

The ability of the endophytic fungi to produce the extracellular enzymes like cellulase and laccase establish their functional role as mutualistic partners with its host plant (Pointing, 1999). The endophytic fungi prove it as saprophytes by exhibiting the capability to produce diverse enzymes without degrading living tissue of the host. The endophytes decompose the dead host plant not only as a single species, but as communities, because invariable species are detected on a single decaying plant part (Sun et al., 2011). The genetic recombination of the endophytes with the host during the evolutionary time could have crooked the endophytes to produce various secondary metabolites as those produced by its associated host (Priest, 1984).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Alves PDD, de Faria Siqueira F, Facchin S, Horta CCR, Victória JMN, Kalapothakis E (2014). Survey of microbial enzymes in soil, water, and plant microenvironments. Open Microbiol. J. 8:25.

Amirita A, Sindhu P, Swetha J, Vasanthi NS, Kannan KP (2012). Enumeration of endophytic fungi from medicinal plants and screening of extracellular enzymes. World J. Sci. Technol. 2(2):13-19.

Barman D, Devadas R (2013). Climate change on orchid population and conservation strategies: a review. J. Crop Weed 9(2):1-12.

Barnett HL, Hunter BB (1998). Illustrated genera of imperfect fungi. American Phytopathological Society. American Phytopathological Society (APS Press). pp. xxii + 218 pp. ISBN: 0890541922

Bayman P, Lebron LL, Tremblay RL, Lodge D (1997). Variation in endophytic fungi from roots and leaves of Lepanthes (Orchidaceae). New Phytol. 135(1):143-149.

Benjamin S, Pandey A (1998). Candida rugosa lipases: molecular biology and versatility in biotechnology. Yeast 14(12):1069-1087.

Benzing DH, Friedman WE (1981). Mycotrophy: its occurrence and possible significance among epiphytic Orchidaceae. Selbyana 5:243 - 247.

Bezerra JDP, Santos MGS, Svedese VM, Lima DMM, Fernandes MJS, Paiva LM, Souza-Motta CM (2012). Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill.(Cactaceae) and preliminary screening for enzyme production. World J. Microbiol. Biotechnol. 28(5):1989-1995.

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004). Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc. R. Soc. Lond. B Biol. Sci. 271(1550):1799-1806.

Boyle C, Gostz M, Dammann-Tugend U, Schulz B (2001). Endophyte – host interactions III. Local vs. systemic colonization. Symbiosis 31:259-281.

Chen J, Zhang LC, Xing YM, Wang YQ, Xing XK, Zhang DW, Liang HQ, Guo SX (2013). Diversity and taxonomy of endophytic xylariaceous fungi from medicinal plants of Dendrobium (Orchidaceae). PloS One 8(3):e58268.

Chen XM, Dong HL, Hu KX, Sun ZR, Chen J, Guo SX (2010). Diversity and antimicrobial and plant-growth-promoting activities of endophytic fungi in *Dendrobium loddigesii* Rolfe. J. Plant Growth Regul. 29(3):328-337.

Choi YW, Hodgkiss IJ, Hyde KD (2005). Enzyme production by endophytes of Brucea javanica. J. Agric. Technol. 1:55-66.

Correa RCG, Rhoden SA, Mota TR, Azevedo JL, Pamphile JA, de Souza CGM, de Moraes, MDLT, Bracht A, Peralta RM (2014).

Endophytic fungi: expanding the arsenal of industrial enzyme producers. J. Ind. Microbiol. Biotechnol. 41(10):1467-1478.
Dearnaley JDW (2007). Further advances in orchid mycorrhizal research. Mycorrhiza 17(6):475-486.
Duncan DB (1955). Multiple range and multiple on decomposition of the leaves of the mangrove F-tests. Biometrics 11:1-42.
Dyk JS, Pletschke BI (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes: factors affecting enzymes, conversion and synergy. Biotechnol. Adv. 30:1458-1480.
El-Bondkly AM, El-Gendy MM (2012). Cellulase production from agricultural residues by recombinant fusant strain of a fungal endophyte of the marine sponge *Latrunculia corticata* for production of ethanol. Antonie Van Leeuwenhoek 101(2):331-346.
Ellis MB (1971). Dematiaceous Hyphomycetes CAB Commonwealth Mycological Institute.
Florencio C, Couri S, Farinas CS (2012). Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by Trichoderma strains. Enzyme Research. Nov 28; 2012.
Hankin L, Anagnostakis SL (1975). The use of solid media for detection of enzyme production by fungi. Mycology 67:597-607.
Hyde KD, Soytong K (2008). The fungal endophyte dilemma. Fungal Divers. 33:163-173.
Illmer P, Schineer F (1995). Solubilization of inorganic calcium phosphate solubilization mechanisms. Soil Biol. Biochem. 27:257-263.
Larran S, Perello A, Simon MR, Moreno V (2002). Isolation and analysis of endophytic microorganisms in wheat (*Triticum aestivum* L.) leaves. World J. Microbiol. Biotechnol. 18(7):683-686.
Magurran AE (1988). Diversity indices and species abundance models. In. Ecological diversity and its measurement . Springer Netherlands. Pp. 7-45.
Maria GL, Sridhar KR, Raviraja NS (2005). Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. J. Agric. Technol. 1:55-66.
Mukul SA, Uddin MB, Tito MR (2007). Medicinal plant diversity and local healthcare among the people living in and around a conservation area of Northern Bangladesh. Int. J. For. Usufructs Manage. 8(2):50-63.
Neha C, Ram P, Ajit V (2015). Plant promoting activities of fungal endophytes associated with tomato roots from central Himalaya, India and their interaction with Piriformospora indica. Int. J. Pharma Bio Sci. 6(1):333-343.
Petrini O, Sieber TN, Toti L, Viret O (1993). Ecology, metabolite production, and substrate utilization in endophytic fungi. Nat. Toxins 1(3):185-196.
Pointing SB (1999). Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Divers. 2:17-33.
Ponmurugan P, Gopi C (2006). Distribution pattern and screening of phosphate solubilizing bacteria isolated from different food and forage crops. J. Argon. 5:600-604.
Pothangbam N, Nirmala C (2011). In vitro Rapid Propagation of *Cymbidium aloifolium* (L.) Sw.: A Medicinally Important Orchid via seed culture. J. Biol. Sci. 11:254-260.
Pridgeon AM, Cribb PJ, Chase MW, Rasmussen F eds (2001). Genera Orchidacearum*:* Volume 2. Orchidoideae. Oxford University Press on Demand.
Priest FG (1984). Extracellular enzymes. Aspect of Microbiology, Volume 9. Van Nostrand Reinhold Wokingham, UK.
Radhika B, Murthy JVVSN, Grace DN (2013).Preliminary phytochemical analysis & antibacterial activity against clinical pathogens of medicinally important orchid *Cymbidium aloifolium* (L.) SW. Int. J. Pharm. Sci. Res. 4(10):3925.
Rajbhandari KR, Dahal S (2004). Orchids of Nepal: A Checklist. Botanica Orientalis 4:89-106.
Rasmussen HN, Whigham DF (2002). Phenology of roots and mycorrhiza in orchid species differing in phototrophic strategy. New Phytol. 154(3):797-807.
Read DJ, Leake JR, Perez-Moreno J (2004). Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Can. J. Bot. 82(8):1243-1263.
Sajben-Nagy E, Manczinge L, Skrbic B, Zivancev J, Antic I, Krisch J, Vagvolgyi C (2014). Characterization of an extracellular laccase of Leptosphaerulina chartarum. World J. Microbiol. Biotechnol. 30(9):2449-2458.
Schulz B, Boyle C, Draeger S, Römmert AK, Krohn K (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol. Res. 106(9):996-1004.
Selosse MA, Faccio A, Scappaticci G, Bonfante P (2004). Chlorophyllous and achlorophyllous specimens of Epipactis microphylla (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb. Ecol. 47(4):416-426.
Smith SE, Read DJ (1997). Mycorrhizal symbiosis, second edition. Academic, San Diego, California. pp. 348-379.
Stone JK, Polishook JD, White JF (2004). Endophytic fungi. Biodiversity of Fungi. Elsevier Academic Press, Burlington. pp. 241-270.
Subedi A, Paudyal G (2001). Some notable orchid of Pokhara valley and their habitats. Botanica Orientalis 2:172-174.
Sun X, Guo L.D, Hyde KD (2011). Community composition of endophytic fungi in Acer truncatum and their role in decomposition. Fungal Divers. 47:85-95.
Sunitha VH, Devi DN, Srinivas C (2013). Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants. World J. Agric. Sci. 9(1):1-9.
Sunitha VH, Ramesha A, Savitha J, Srinivas C (2012). Amylase production by endophytic fungi Cylindrocephalum sp. isolated from medicinal plant *Alpinia calcarata* (Haw.) Roscoe. Braz. J. Microbiol. 43:1213-1221.
Sutton BC (1980). The coelomycetes. CMI. Kew, Surrey, England.
Takahashi K, Ishikawa H, Ogino T, Hatana, T, Ogiwara I (2007). Growth assay of daughter tubers from the tubers of Habenariaradiata (Thunb) K. Spreng. Seedlings gel covered and inoculated with orchid mycorrhizal fungi in habitat. Hortic. Res. (Japan) 6:33-36.
Tao G, Liu ZY, Hyde KD, Lui XZ, Yu ZN (2008). Whole rDNA analysis reveals novel and endophytic fungi in Bletilla ochracea (Orchidaceae). Fungal Divers. 33:101-122.
Uzma F, Konappa NM, Chowdappa S (2016). Diversity and extracellular enzyme activities of fungal endophytes isolated from medicinal plants of Western Ghats, Karnataka. Egypt. J. Basic Appl. Sci. 3(4):335-342.
Venkatesagowda B, Ponugupaty E, Barbosa AM, Dekker RF (2012). Diversity of plant oil seed-associated fungi isolated from seven oil-bearing seeds and their potential for the production of lipolytic enzymes. World J. Microbiol. Biotechnol. 28(1):71-80.
Ward OP (2012). Production of recombinant proteins by filamentous fungi. Biotechnol. Adv. 30(5):1119-1139.
Wei DL, Kirimura K, Usami S, Lin TH (1996). Purification and characterization of an extracellular β-glucosidase from the wood-grown fungus Xylaria regalis. Curr. Microbiol. 33(5):297-301.
Zaferanloo B, Virkar A, Mahon PJ, Palombo EA (2013). Endophytes from an Australian native plant are a promising source of industrially useful enzymes. World J. Microbiol. Biotechnol. 29(2):335-345.
Zhu GS, Yu ZN, Gui Y, Liu ZY (2008). A novel technique for isolating orchid mycorrhizal fungi. Fungal Divers. 33:123-137.

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