Fatima, I., Jamil, M., Hussain, A., Mumtaz, M. Z., Luqman, M., Hussain, S., Kashif, S. R., & Ahmad, M.  
Zinc solubilizing Bacillus sp. ZM20 and Bacillus aryabhattai ZM31 promoted the productivity in okra (Abelmoschus esculentus L.)  

Aslam, M., Noreen, Z., Asghar, A., Hussain, A., Mehmood, R., Khan, M. U., Saleem, K., Hussain, M. T., & Chaudhary, A.  
One-pot unusual synthesis and DFT studies of 3-acetyl-6-chloro-2-methyl-4-phenylquinoline and its unusual urease enhancement activity  

Shahzad, L., Tahir, A., & Sharif, F.  
Understanding the community’s perception of climate change and adaptations in the Mid Hills of Pakistan  

Iqbal, T., Rashid, U., & Idrees, M.  
Structure prediction of ORF3 encoded protein of a novel Pakistani avian hepatitis E virus strain  

Haider, S. M., Bhatti, K. H., Siddiqi, E. U., Irfan, S. M., & Gulshan, A. B.  
Estimation of antibacterial action of Aloe Vera L. on different strains and concentrations  

Shahid, M., Jabeen, K., & Iqbal, S.  
Evaluation of antifungal potential of Wood Biochar against Fusarium oxysporum Schlecht  

Saeed, Z., Iqbal, S., Younas, U., Pervaiz, M., Naqvi, S. M. A., & Khan, R. R. M.  
Optimization of sewage sludge mixing with soil to attain promising neutrautectual attributes in the Lagenaria siceraria L. (Kaddu) Plant  

Sial, Z. K., & Khan, F.  
First report on Potato Spindle Tuber Viroid (Pstvd) from field grown infected Potato Plants (Solanum Tuberosum) in Pakistan  

Afifab, M., Tahir, A., Asim, T., & Maryam, I.  
Optimization of cultural conditions for enhanced production of laccase by Aspergillus flavus Maf 0139  

Sugar Industry effluents as a source of Soil Fertility and Potential Toxicological risk of heavy metals in food crop  

Investigation of Nickel in soil, forages and blood plasma of buffaloes with respect to seasonal variations  

Mubeen, H., Naseem, A., Masood, A., Raza, S., & Naeem, N.  
Cis-acting regulatory elements and transcription factors as a key regulator in plant gene expression  

Iqbal, I., Tanweer, P., Manzoor, F., Afifab, M. N., Kaleem, A., Abdullah, R., Zafar, A., & Iqtedar, M.  
Antimicrobial properties of Dalbergia, Brassica and Trifolium honey against burn Microorganisms  

Analysis of biological potential of Aromatic Hydrazones as Novel Therapeutic Agents  

Malkani, N., Ishaque, I., Shahbaz, K., Khan, R. U., Shabbir, A., & Yaqub, A.  
Health comparison of farm-raised and wild-caught Labeo rohita (Cypriniformes: Cyprinidae)  

Mahmood, A., Sajid, Z. A., & Khilji, S. A.  
Influence of Salicylic Acid on salinity stress tolerance by seed priming and foliar application on Maize (Zea Mays)
Zinc solubilizing Bacillus sp. ZM20 and Bacillus aryabhattachai ZM31 promoted the productivity in Okra (Abelmoschus esculentus L.)

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ABSTRACT

Zinc solubilizing bacteria (ZSB) improve crop productivity by increasing bioavailability of zinc (Zn). A pot experiment was conducted to evaluate the effectiveness of five promising ZSB strains on the productivity of okra. The experiment was conducted using Completely Randomized Design (CRD) with four replications. Data regarding physiological, growth, and yield parameters were collected and statistically analyzed. Results showed that inoculation of ZSB strains significantly increased these attributes of okra. Inoculation of strain Bacillus sp. ZM20 followed by Bacillus aryabhattachai ZM31 was significantly more effective among the tested ZSB strains. Strain Bacillus sp. ZM20 improved relative water contents up to 17%, chlorophyll a and b up to 67 and 71%, respectively, plant height up to 30%, shoot fresh weight up to 19%, shoot dry weight up to 31%, root length up to 79%, root fresh weight up to 58%, root dry weight up to 66%, number of fruits plant-1 up to 89%, fruit fresh weight up to 79%, fruit dry weight up to 78%, concentration of N up to 20%, P up to 65%, K up to 20%, and protein contents up to 20% as compared to uninoculated control. It is concluded that inoculation of ZSB strains like Bacillus sp. ZM20 and Bacillus aryabhattachai ZM31 is an effective approach to improve the productivity of okra (Abelmoschus esculentus L.).

Keywords: Okra (Abelmoschus esculentus L.), Bacillus strains, Nutrient solubilization, Plant nutrition, Sustainable

INTRODUCTION

Okra (Abelmoschus esculentus L.) is widely consumed vegetable crop. It provides high nutritional contents such as carbohydrates, minerals, proteins, calcium, iron and vitamins to human diet (Bawa & Badrie, 2016). In Pakistan, 13.9 thousand hectares area is under okra cultivation with a production of 102.6 thousand tons (Khokhar, 2014). Its production is very low in most developing countries including Pakistan because of its reliance on natural soil fertility.

Low solubility of Zn in soils is an important factor for reducing crop yield and production as it plays important role in metabolism of nucleic acid, cell division, synthesis of proteins and synthesis of indole acetic acid (MacDonald, 2000; Rout & Das, 2009). Zn deficiency occurs in 70% soils of Pakistan due to calcareous nature, low organic matter and high pH and causes crop failure (Bapiri et al., 2012). Low Zn solubility in soils is the main cause of Zn deficiency in crops rather than a low total Zn contents (Cakmak, 2008; Alloway, 2009). Its low availability decreases the yield and leads to the inferior quality of crop products and is responsible for its deficiency in humans (Rehman et al., 2018).

Zinc deficiency in rhizosphere is being corrected via use of manures and chemical fertilizers. Chemical fertilizers enhance the fertility status of soils and productivity of crops but these also affect the soil chemistry negatively and are very costly (Steinshamn et al., 2004). Application of manure fulfills the Zn requirements but depends on the factors like soil physico-chemical properties, temperature, moisture, characteristics of manure, and microbial activity in soil (Alloway, 2009). Researcher reported the increase in crop production and quality of food produced by use of rhizobacteria. They colonize in rhizosphere and increase the plant growth through number of primary and secondary metabolites involved in solubility of phosphorus (P), potassium (K), Zn, iron (Fe), biological nitrogen (N) fixation, production of...
siderophores, syntheses of phytohormones and control of plant pathogens (Freitas et al., 2007; Lugtenberg & Kamilova, 2009; Mumtaz et al., 2017).

Most of soils contain significant Zn concentration in unavailable forms which can be converted to available form by inoculation of Zn solubilizing bacterial (ZSB) strains (Saravanan et al., 2004; Bapiri et al., 2012; Mumtaz et al., 2017, 2018; Khanghahi et al., 2018). They dissolve the insoluble Zn compounds via producing organic acids like gluconic acid and 2-keto gluconic acids (Bapiri et al., 2012). Inoculation of ZSB in rhizosphere enhances the concentration of Zn and decreases the dependence on synthetic fertilizers. Unwise and indiscriminate use of perilous agricultural chemicals can be reduced by inoculation of ZSB which are good substitute of chemicals for increasing the growth and yield of plants (Vessey, 2003). Therefore, keeping in view the above scenario, the present study was conducted to evaluate the impact of ZSB strains on growth, physiology and productivity of okra.

MATERIALS AND METHODS

Collection of bacterial strains and preparation of inoculum

Five ZSB strains viz. ZM19, ZM20, ZM27, ZM31, and ZM50 were obtained from gene bank of Soil Microbiology and Biotechnology Laboratory, Department of Soil Science, The Islamia University of Bahawalpur. These strains were previously characterized, screened, and evaluated for plant growth promotion by Mumtaz et al. (2017, 2018). Among these tested strains, strains ZM20 and ZM31 were identified as Bacillus sp. ZM20 and Bacillus aryabhattai ZM31 (Mumtaz et al., 2017). The bacterial cultures were grown in DF-minimal salt broth amended with 0.1% zinc oxide (ZnO) in shaking incubator (Model S19R-2, Shellab-USA) for 48 h. After incubation, bacterial cultures were maintained to uniform population (cell count of $10^8$ cfu ml$^{-1}$) and used for seed inoculation.

Seed inoculation and experimental management

Seeds of okra variety Sabz Pari was purchased from local seed market of Bahawalpur and sterilized by following method of Khalid et al. (2004) and dipped in the respective bacterial culture for 30 mints before seed sowing. Whereas, the control seeds were dipped in broth. Pot experiment was performed at the wire house of Department of Soil Science, The Islamia University of Bahawalpur, Pakistan, located at Lat: 29.40N, Lon: 71.68E and 116 meters elevation above the sea level. Pots were filled with 12 kg sieved loamy soil. Inoculated seeds were sown in pots arranged in Completely Randomized Design (CRD) having four replicates. Recommended doses of N, P and K (50: 25: 25 kg ha$^{-1}$) were applied in the form of Urea, Diammonium Phosphate (DAP) and Muriate of Potash (MOP), respectively. Full P and K were applied at sowing time while N was applied in three splits doses: first dose at sowing and remaining at 15 days interval. All the recommended agronomic practices were carried out. At physiological maturity, data regarding physiological attributes were recorded while at harvesting; growth and yield parameters were estimated.

Plant analysis

At flowering stage, relative water content (RWC) of top fully developed okra leaf was determined by using formula described by Mayak et al. (2004). Chlorophyll a and b contents were also determined spectrophotometrically and values were calculated by method of Arnon (1949). Okra fruits were harvested at marketable stage and biometrical observation like number of fruits plant$^{-1}$, fresh and dry weight of fruits were recorded.

For chemical analysis, 100 g of mix okra shoot and leaf were dried in an oven at 65 °C to constant weight and grounded into powder. Plant samples were digested as described by Wolf (1982). The N contents in plant samples were determined by Kjeldal method while P concentration was estimated through adopting procedure of Jackson (1973). The K concentration was determined through flame photometer model BWB-XP (BWB technology Ltd. UK). Values were compared with calibration curve of KCl standard ranging from 0 to 100 ppm and actual concentration was calculated.

The data of various attributes was analyzed for analysis of variance techniques (ANOVA) in accordance with CRD design and means were compared by least significant difference (LSD) test at 5% probability (Steel et al., 2007).

RESULTS

Physiological parameters

Inoculation with ZSB strains significantly increased RWC and chlorophyll a' and b' contents (Table I). Inoculation with strain ZM20 reported maximum increase up to 16.8% in RWC as compared to uninoculated control. The strain ZM31 also showed better increase up to 4.2% and was non-significant to strains ZM19, ZM27, and ZM50.
but significantly different from uninoculated control. The maximum chlorophyll ‘a’ and ‘b’ contents with an increase up to 66.7 and 70.6%, respectively, were observed due to strains ZM20 followed by strain ZM31 that gave 54.5 and 61.8% more chlorophyll a and b contents, respectively, over uninoculated control.

Table I: Effect of zinc solubilizing bacteria on relative water contents, chlorophyll ‘a’ and chlorophyll ‘b’ contents of okra leaves

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Relative water contents (%)</th>
<th>Chlorophyll ‘a’ (µg/g)</th>
<th>Chlorophyll ‘b’ (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>68.46 c</td>
<td>0.99 d</td>
<td>1.02 f</td>
</tr>
<tr>
<td>ZM19</td>
<td>69.63 bc</td>
<td>1.39 c</td>
<td>1.57 c</td>
</tr>
<tr>
<td>ZM20</td>
<td>79.98 a</td>
<td>1.65 a</td>
<td>1.74 a</td>
</tr>
<tr>
<td>ZM27</td>
<td>69.71 bc</td>
<td>1.32 c</td>
<td>1.50 d</td>
</tr>
<tr>
<td>ZM31</td>
<td>71.48 ab</td>
<td>1.53 b</td>
<td>1.65 b</td>
</tr>
<tr>
<td>ZM50</td>
<td>70.65 ab</td>
<td>1.05 d</td>
<td>1.39 e</td>
</tr>
<tr>
<td>LSD (p≤0.05)</td>
<td>1.5517</td>
<td>0.0752</td>
<td>0.0582</td>
</tr>
</tbody>
</table>

Means sharing different letters are statistically significant from each other at 5% level of probability (n = 4)

Table II: Effect of zinc solubilizing bacteria on plant height, shoot fresh and dry weight of okra

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant height (cm)</th>
<th>Shoot fresh weight (g plant⁻¹)</th>
<th>Shoot dry weight (g plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.19 f</td>
<td>125.32 f</td>
<td>75.74 f</td>
</tr>
<tr>
<td>ZM19</td>
<td>93.90 c</td>
<td>138.91 c</td>
<td>91.59 c</td>
</tr>
<tr>
<td>ZM20</td>
<td>104.03 a</td>
<td>149.26 a</td>
<td>99.29 a</td>
</tr>
<tr>
<td>ZM27</td>
<td>90.28 d</td>
<td>134.39 d</td>
<td>85.21 d</td>
</tr>
<tr>
<td>ZM31</td>
<td>98.66 b</td>
<td>145.87 b</td>
<td>96.93 b</td>
</tr>
<tr>
<td>ZM50</td>
<td>86.53 e</td>
<td>130.44 e</td>
<td>81.17 e</td>
</tr>
<tr>
<td>LSD (p≤0.05)</td>
<td>2.5505</td>
<td>2.4762</td>
<td>2.0172</td>
</tr>
</tbody>
</table>

Means sharing different letters are statistically significant from each other at 5% level of probability (n = 4)

Table III: Effect of zinc solubilizing bacteria on root length, root fresh and dry weight of okra

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root Length (cm)</th>
<th>Root fresh weight (g plant⁻¹)</th>
<th>Root dry weight (g plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44.02 f</td>
<td>59.75 f</td>
<td>29.41 f</td>
</tr>
<tr>
<td>ZM19</td>
<td>64.13 c</td>
<td>80.75 c</td>
<td>38.56 c</td>
</tr>
<tr>
<td>ZM20</td>
<td>78.77 a</td>
<td>94.50 a</td>
<td>48.83 a</td>
</tr>
<tr>
<td>ZM27</td>
<td>61.07 d</td>
<td>76.75 d</td>
<td>35.36 d</td>
</tr>
<tr>
<td>ZM31</td>
<td>72.80 b</td>
<td>89.25 b</td>
<td>42.82 b</td>
</tr>
<tr>
<td>ZM50</td>
<td>53.80 e</td>
<td>71.50 e</td>
<td>32.16 e</td>
</tr>
<tr>
<td>LSD (p≤0.05)</td>
<td>1.7231</td>
<td>1.8856</td>
<td>1.2940</td>
</tr>
</tbody>
</table>

Means sharing different letters are statistically significant from each other at 5% level of probability (n = 4)

Table IV: Effect of zinc solubilizing bacteria on number of fruits plant⁻¹, fresh and dry weight of okra fruit

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of fruits Plant⁻¹</th>
<th>Fruit fresh weight (g)</th>
<th>Fruit dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.5 e</td>
<td>66.09 f</td>
<td>11.45 e</td>
</tr>
<tr>
<td>ZM19</td>
<td>13.6 b</td>
<td>97.05 c</td>
<td>15.09 c</td>
</tr>
<tr>
<td>ZM20</td>
<td>16.0 a</td>
<td>118.03 a</td>
<td>20.49 a</td>
</tr>
<tr>
<td>ZM27</td>
<td>12.2 c</td>
<td>94.25 d</td>
<td>13.94 d</td>
</tr>
<tr>
<td>ZM31</td>
<td>14.8 b</td>
<td>110.11 b</td>
<td>16.99 b</td>
</tr>
<tr>
<td>ZM50</td>
<td>10.9 d</td>
<td>87.90 e</td>
<td>13.53 d</td>
</tr>
<tr>
<td>LSD (p≤0.05)</td>
<td>0.0264</td>
<td>1.4779</td>
<td>0.9722</td>
</tr>
</tbody>
</table>

Means sharing different letters are statistically significant from each other at 5% level of probability (n = 4)
Table V: Effect of zinc solubilizing bacteria on NPK and protein % age in okra

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrogen (%)</th>
<th>Phosphorus (%)</th>
<th>Potassium (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.18 e</td>
<td>0.26 d</td>
<td>1.50 f</td>
<td>13.63 e</td>
</tr>
<tr>
<td>ZM19</td>
<td>2.42 c</td>
<td>0.34 c</td>
<td>1.76 c</td>
<td>15.13 c</td>
</tr>
<tr>
<td>ZM20</td>
<td>2.62 a</td>
<td>0.43 a</td>
<td>1.80 a</td>
<td>16.37 a</td>
</tr>
<tr>
<td>ZM27</td>
<td>2.38 c</td>
<td>0.33 c</td>
<td>1.71 d</td>
<td>14.91 c</td>
</tr>
<tr>
<td>ZM31</td>
<td>2.46 b</td>
<td>0.39 b</td>
<td>1.82 b</td>
<td>15.40 b</td>
</tr>
<tr>
<td>ZM50</td>
<td>2.31 d</td>
<td>0.27 d</td>
<td>1.63 e</td>
<td>14.48 d</td>
</tr>
<tr>
<td>LSD (p≤0.05)</td>
<td>0.0414</td>
<td>0.0196</td>
<td>0.0264</td>
<td>0.2577</td>
</tr>
</tbody>
</table>

Means sharing different letters are statistically significant from each other at 5% level of probability (n = 4)

Agronomic parameters

Results showed that inoculation of ZSB strains was effective in improving agronomic attributes in terms of plant height, shoot fresh and dry weight, root length, and root fresh and dry weight as compared to uninoculated control (Table II). The maximum increase in plant height, shoot fresh and dry weight was given by strain ZM20 with increase up to 29.7, 19.1, and 31.1%, respectively, followed by ZM30 that improved these attributes up to 23.0, 16.4, and 28.0%, respectively, over uninoculated control.

Significant variation in root growth in terms of root length, root fresh and dry weight was observed in as inoculated as compared to uninoculated control (Table III). Inoculation of ZM20 reported maximum increase up to 78.9, 58.2, and 66.0%, in root length, root fresh and dry weight, respectively, of okra plants as compared to uninoculated control. Inoculation of strain ZM31 were also able to show better root length, root fresh and dry weight with increase up to 65.4, 49.4, and 45.6%, respectively, over uninoculated control.

Yield parameters

Among yield contributing attributes of okra, number of fruits plant\(^{-1}\) were significantly promoted due to inoculation with ZSB strains (Table IV). Uninoculated control reported minimum number of fruits plant-1 which were 8.5. Among inoculation treatment, strain ZM20 reported maximum number of fruits plant\(^{-1}\) over uninoculated control which were 16, while strain ZM50 gave poor number of fruits plant\(^{-1}\) however significantly different from uninoculated control. Data regarding the effect of ZSB strains on okra fruit fresh and dry weight (Table IV) showed that fruit biomass was improved due to inoculation. The maximum fruit fresh and dry weights were observed due to strain ZM20 being 118.0 and 20.5 g plant\(^{-1}\), respectively, followed by the inoculation with ZM31 that showed 110.1 and 16.9 g plant\(^{-1}\) of fruit fresh and dry weight, respectively. Minimum fruit fresh and dry weights were observed from uninoculated control.

NPK and protein contents

The improvement in NPK and Protein concentration in straw was observed due to inoculation treatments (Table V). The inoculation of strain ZM20 increased N concentration up to 20.2% as compared to uninoculated control. The maximum increase in P, K, and protein concentration up to 65.0, 20.0, and 20.1%, respectively was also observed by strain ZM20 as compared to uninoculated control.

DISCUSSION

Zinc solubilizing bacteria (ZSB) can promote crop productivity through improving soil fertility. These microbes improved plant health under normal as well as environmental stress conditions and reduced the dependence on hazardous chemicals. In the present study, ZSB strains improved the physiological attributes like RWC, chlorophyll ‘a’ and chlorophyll ‘b’ contents of okra plants. Improvement in RWC might be due to increase in root surface area that enhanced water uptake. These results were supported by Ahmad et al. (2011) who described that co-inoculation of rhizobacterial and rhizobial strains improved root length that helped water uptake from depth. Similarly, Egamberdiyeva (2007) and Mumtaz et al. (2018) also found that application of rhizobacteria improved root length and root surface area that increased water uptake from far places and resulted in improvement of relative water content. Nayak et al. (1986), stated that plants inoculated with PGPR showed increase in chlorophyll contents and photosynthetic rate which led to overall improvement in plant health. Increase in chlorophyll a and b was similar to findings of Sharma et al. (2003) who reported the increased in chlorophyll ‘a’ and chlorophyll ‘b’ in rhizobacterial inoculated plants that resulted in increased growth and yield.

The present study showed that inoculation of ZSB strains significantly improved the growth of okra. It could be due to the ability of bacterial strains to create favorable conditions for vegetative
growth and to increase shoot and root growth by making nutrients more available to the roots (Adesemoye & Ugoji, 2006). Han et al. (2007) studied that soil microbes used as bio-fertilizers play vital functions in decaying organic matter, nutrient cycling and supporting crop growth and health. Richardson (2001) described that rhizobacterial inoculation efficiently increased the root surface area and biomass due to more production of phytohormones by bacterial strains that facilitated more nutrient absorption. Current study also reported the increase in fruit biomass due to ZSB strains which was similar to the findings of Jayapandi & Balakrishnan (1990) who reported the increase in yield component of okra as a result of application of rhizobacterial strains. It is well-documented that biofertilizer enhanced plant growth and yield through making nutrient more available and improving soil health (Iqbal et al., 2013).

Inoculation of ZSB strains increased the NPK and protein contents as compared to uninoculated control under current study. The increase in nutrient concentration in plants could be due to their effect on initiation and development of lateral roots and increased root weight. Ahmad et al. (2014) reported the increase in root surface area through root proliferation as a result of bacterial strains inoculation which were responsible for the availability of nutrients. The secretion of acids by bacteria and other behavior of soil microbiota affect the equilibrium towards more nutrient solubility and bioavailability to plant roots for absorption (Saravanan et al., 2004). Similarly Estrada et al. (2013) and Abaid-Ullah et al. (2015) evaluated the bacterial strains for secretions of organic acids in response of insoluble nutrient like P, K, and Zn and reported the production of gluconic, oxalic, citric, malic acids, etc. These organic acids have the power to acidified the soil medium and solubilize insoluble compounds. Thus, Zn solubilizing bacterial strains in present work improved the productivity of okra through improving physiology, growth, and yield and increasing the accumulation of nutrients in okra.

**CONCLUSION**

Inoculation of zinc solubilizing bacterial strains significantly improved the physiological, growth, yield attributes and nutrients concentration in okra. The strains *Bacillus* sp. ZM20 followed by *Bacillus aryabhattai* ZM31 showed more promising results. These strains are well-capable to convert unavailable forms of nutrients into available forms which is unconventional tool to lessen the nutrients deficiency in plant and produce superior quality plant products. These strains could also be better substitute for farmers to lessen the application of chemical fertilizers for sustainable production of crops.

**ACKNOWLEDGEMENT**

We are thankful to Department of Soil Science, University College of Agriculture and Environmental Sciences, the Islamia University of Bahawalpur for provision of research facilities for this study.

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One-pot unusual synthesis and DFT studies of 3-acetyl-6-chloro-2-methyl-4-phenylquinoline and its unusual urease enhancement activity

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ABSTRACT

A surprising synthesis of 3-acetyl-6-chloro-2-methyl-4-phenylquinoline has been attained through convenient and efficient one-pot condensation of 3-chlorobenzophenone and acetylacetone. Its structure was elucidated by spectral data, X-ray diffraction studies and also investigated using density functional theory (DFT). Optimized geometry was obtained by performing DFT calculations at B3LYP level of theory and 6-311+G (d,p) basis set. Frontier molecular orbital analysis has been executed at B3LYP/6-311+G(d,p) level of theory. The global reactivity parameters were explored using the energy of frontier molecular orbitals. Natural bond orbital analysis has been carried out at B3LYP/6-311+G(d,p) level of theory to discover hyper conjugative interaction and stability of the title molecule. Moreover, the product showed the reverse urease inhibition activity.

Keywords: Quinoline derivative: Schiff base: Urease inhibition activity: Density functional theory: Natural Bond Orbital: Frontier Molecular Orbital

INTRODUCTION

The diverse biological uses of Schiff bases engrossed us to synthesize some new Schiff bases (Andiappan et al., 2018; Jawoor et al., 2017). In this struggle, we have reported synthesis, X-ray structures and biological activities of various Schiff bases (Aslam et al., 2012). In the present study, we planned to synthesize a Schiff base from 3-chlorobenzophenone and acetylacetone by the same previously used procedure but surprisingly a quinoline compound was obtained instead of the target product. Quinolines are nitrogen containing heterocyclic aromatic compounds and have diverse pharmacological activities such as antipyretic, antimalarial, analgesic, anti-inflammatory, arthritis, intracellular singling, antibiotic resistance and food preservatives etc. So herein we report the synthesis, characterization and urease activity of 3-acetyl-6-chloro-2-methyl-4-phenylquinoline. Urease activity results showed that the product enhanced the activity instead of inhibition. The urease enzyme causes the gastric ulceration, urinary stone formation, pyelonephritis, and other dysfunctions (Moncrief et al., 1995). Furthermore, the synthesized compound was subjected to density functional theory (DFT) calculations to explore more about molecular geometry, electronic properties such as frontier molecular orbitals (FMOs) and natural bond orbital (NBO) analysis.
MATERIALS AND METHODS

All the chemicals and solvents were purchased from E. Merck. TLC was performed on pre-coated silica gel G-25-UV\(\text{254}\) plates (E. Merck), and detection was carried out at 254 and 366 nm. The IR spectrum was recorded on Thermo Nicolet Avatar 320 FTIR spectrometer using KBr pellets. Melting points were recorded on a Gallenkamp apparatus. Elemental analysis was performed on Perkin Elmer 2400 Series II elemental analyzer. The FAB mass spectrum was recorded on JEOL SX102/DA-6000 mass spectrometer using glycerol as matrix and ions are given in \(m/z\) (%). The NMR (\(^1\)H, \(^{13}\)C, 2D) spectra were recorded on a Bruker AMX-400 spectrometer in DMSO-\(d_6\). The chemical shifts (\(\delta\)) are given in ppm, relative to tetramethylsilane as an internal standard, and the scalar coupling constants (\(J\)) are reported in Hertz. Single-crystal X-ray diffraction data was collected on Bruker Smart APEX II, CCD 4-K area detector diffractometer (Siemens 1996). Data reduction was performed by using SAINT program. The structure was solved by direct method (Altomare et al., 1993), and refined by full-matrix least squares on \(F^2\) by using the SHELXTL-PC package (Siemens 1997). The figures were plotted with the aid of ORTEP program (Johnson 1976).

Procedure of the synthesis

The mixture of 2-amino-5-chlorobenzophenone (0.01 mole, 2.31 g) and pentane-2,4-dione (acetylacetone) (0.01 mole, 1.00 g) in ethanol (50 mL) followed by 3-4 drops of conc. \(\text{H}_2\text{SO}_4\) was refluxed for 7 h at 70°C (see synthetic scheme at Fig. 1). After cooling, the reaction mixture was concentrated to one third of its volume by rotary evaporator. The concentrated mixture was kept at room temperature for five days and white transparent crystals were obtained. The crystalline product was collected, washed with methanol and dried to afford the pure product in 87% yield. Purity of the product was checked by TLC.

Fig. 1: Synthetic scheme of 3-Acetyl-6-chloro-2-methyl-4-phenylquinoline (3).

Urease inhibition assay

The urease enzyme solution was prepared by taking 0.125 units in each well in phosphate buffer (\(K_2\)HPO\(_4\), 3H\(_2\)O, 1 mM EDTA and 0.01M LiCl\(_2\)). Each well was filled with 80 \(\mu\)L of 0.05 M potassium phosphate buffer (pH 8.2), 10 \(\mu\)L of the sample (concentration range 5 - 500 \(\mu\)M), contents were mixed and incubated for 15 min at 30°C. 40 Microliter of substrate solution (urea, 50 mM) was poured in each well to initiate reaction. Then, 70 \(\mu\)L alkaline reagent (0.5 % NaOH and 0.1 % active \(\text{NaOCl}\)) and 40 \(\mu\)L of phenol reagent (1 % phenol and 0.005 % w/v sodium nitroprusside) were introduced to each well. The well plate, containing reaction mixture, was incubated for 50 minutes and absorbance was recorded at 630 nm. IC\(_{50}\) values were determined by monitoring the effect of increasing concentrations of the product on extent of inhibition.

Computational procedure

Gaussian 09 program package (Frisch et al., 2016) employing density functional theory (DFT) (Braga et al., 2005) was used to perform whole computations. The initial geometry of the title molecule was retrieved from the crystal structures. Full optimization was carried out without using symmetry restrictions at B3LYP level of theory and 6-311+G(d,p) basis set. All vibrational frequencies calculated ascertain the structure is stable as no imaginary frequencies were observed. FMO and NBO analysis were performed using B3LYP level of theory with 6-311+G(d,p) basis set combination. The softwares Gauss View 5.0 (Frisch et al., 2000), Avogadro (http://avogadro.cc/wiki/Main_Page) and Chem Craft (http://www.chemcraftprog.com) were used to interpret the output files results.

RESULTS AND DISCUSSION

The product, 3-acetyl-6-chloro-2-methyl-4-phenylquinoline 3, was synthesized in good yield by the double condensation of 3-chlorobenzophenone 1 and acetylacetone 2 in the presence of few drops of conc. sulfuric acid as catalyst. The IR spectrum of the product exhibited the absorption bands at 1705 and 1610 cm\(^{-1}\), which is typical of Schiff bases (azomethine moiety) (Nicolae and Anghel 2003), indicating the product is not the expected Schiff base. The \(^1\)H NMR spectrum showed the aromatic methine protons signals at \(\delta\) 8.05 (1H, d, \(J = 9.2\) Hz, H-8), 7.80 (1H, dd, \(J = 9.2, 2.4\) Hz, H-7) and 7.39 (1H, d, \(J = 2.4\) Hz, H-5). The protons of the mono-substituted benzene ring resonated at \(\delta\) 7.57-
7.60 (3H, m, H-3', -4', -5') and 7.36 (2H, dd, J = 6.4, 2.0 Hz, H-2', -6'). The spectrum also showed two methyl singlets at δ 2.59 and 2.04, and their chemical shifts indicating their attachment with the aromatic ring and the carbonyl carbon, respectively. The absence of the methylene protons signal in the spectrum revealing that expected Schiff base product was not formed and may be methylene has been used in ring formation. The $^{13}$C NMR spectrum showed a signal of the carbonyl carbon at δ 204.4 and did not show the signal of the methylene carbon. If expected Schiff base product formed then the signals of the second carbonyl and the methylene carbons must be present in the spectrum. It can be expected that one carbonyl and the methylene has been utilized in the third ring formation. In the HMBC experiment the protons at δ 7.36 (H-2', -6') showed $^3$J correlation with the quarternary carbon at δ 142.2. It is further evidence that the carbonyl of 3-chlorobenzophenone has become the part of aromatic ring. All the HMBC and COSY correlations are shown in the Fig., 2.

![Fig. 2: All HMBC (→) and COSY (—) correlations of the product](image1)

EIMS showed the [M]$^+$ peak at m/z 295 for the molecular formula C$_{18}$H$_{13}$ClNO. It is also confirming that second H$_2$O molecule has also been removed by second condensation. Elemental analysis also fully supported the molecular formula. On the basis of all spectral data, it is evident that a double condensation has taken place during the reaction. Finally, X-ray diffraction confirmed the product structure (Fig., 3 and 4).

![Fig. 3: The structure of the product with displacement ellipsoids drawn at 30% probability level](image2)

![Fig. 4: The crystal packing of the product](image3)
3-Acetyl-6-chloro-2-methyl-4-phenylquinoline (3)
White crystalline solid; m.p. 154-155 °C; yield 87 %; IR (KBr) ν max cm⁻¹: 1705 (C=O), 2923 (C-H), 1606-1415 (aromatic moiety), 700 (C -Cl); 1H and 13C NMR: See Table I; EI -MS (70 e/v) m/z (rel. int %): 295 (45, [M]+), 280 (100), 252 (22), 217 (20), 176 (17); FAB-MS (+ve): 296 [M+H]+ (calcd for C18H14ClNO, 296); Anal Calc. for C18H14ClNO: C 73.10; H 4.77; N 4.74. Found: C 73.15; H 5.84; N 4.70.

Table I: 1H (400 MHz, DMSO-d6) and 13C (100 MHz, DMSO-d6) NMR data of the product

<table>
<thead>
<tr>
<th>C No.</th>
<th>C Type</th>
<th>δC</th>
<th>δH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>C</td>
<td>153.9</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>135.3</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>142.4</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>CH</td>
<td>124.2</td>
<td>7.39 (1H, d, J = 2.4 Hz)</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>131.3</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
<td>CH</td>
<td>130.7</td>
<td>7.80 (1H, dd, J = 9.2, 2.4 Hz)</td>
</tr>
<tr>
<td>8</td>
<td>CH</td>
<td>130.8</td>
<td>8.05 (1H, d, J = 9.2 Hz)</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>125.4</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td>145.2</td>
<td>---</td>
</tr>
<tr>
<td>1'</td>
<td>C</td>
<td>133.9</td>
<td>---</td>
</tr>
<tr>
<td>2', 6'</td>
<td>CH</td>
<td>129.7</td>
<td>7.36 (2H, dd, J = 6.4, 2.0 Hz)</td>
</tr>
<tr>
<td>3', 5'</td>
<td>CH</td>
<td>128.9</td>
<td>7.57-7.60 (2H, m)</td>
</tr>
<tr>
<td>4'</td>
<td>CH</td>
<td>129.3</td>
<td>7.57-7.60 (1H, m)</td>
</tr>
<tr>
<td>1''</td>
<td>C</td>
<td>204.7</td>
<td>---</td>
</tr>
<tr>
<td>2''</td>
<td>CH3</td>
<td>31.7</td>
<td>2.04 (3H, s)</td>
</tr>
<tr>
<td>1'''</td>
<td>CH3</td>
<td>23.4</td>
<td>2.59 (3H, s)</td>
</tr>
</tbody>
</table>

X-Ray diffraction of the product
Single-crystal X-Ray diffraction analysis was carried out to establish the structure of the product. The ORTEP diagram (Fig., 3) of the product showed that the quinoline ring (C1–C9/N1) is approximately planar, with a maximum deviation of 0.017 (4) Å for atom C4 and form a dihedral angle of 64.40(14)° with the mean plane of the phenyl ring (C10–C15). All bond angles and lengths were found to be in normal range (Allen et al., 1987). In crystal structure no classical hydrogen bonding was found and molecules are arranged in zig zag fashion parallel to the a-axis (Fig., 4).

X-Ray crystal data of the product
C18H14ClNO1, Mr = 295.75, monoclinic, space group P21/n, a = 10.4798(13) Å, b = 8.0045(10) Å, c = 17.562(2) Å, β = 90.764(3)°, V= 1473.1(3) Å³, Z=4, ρcalc = 1.334 mg/m³, F(000) = 616, μ(Mo Kα = 0.71073 Å, max/min transmission 0.9723 / 0.9110, crystal dimensions 0.37 x 0.29 x 0.11, 2.25° < θ< 25.5°, 7930 reflections were collected, of which 2561 reflections were observed (Rint = 0.0411). The R values were: R1 = 0.0591, wR2 = 0.1591 for I > 2σ(I), and R1 = 0.0902, wR2 = 0.1703 for all data; max/min residual electron density: 0.222 / -0.183 e A⁻³. Crystallographic data of the product has been deposited in the Cambridge Crystallographic Data Center. The crystallographic information can directly be obtained free of charge from CCDC data center (CCDC 883325 reference code).

Urease enhancement activity
During the urease inhibition studies of the product, it was found that it enhanced the activity instead of inhibition. To check how much it enhances, thiourea was used as standard, which showed 96 % inhibition against urease enzyme. For this, first, the product was incubated with urease for two hours and then thiourea was added and
measured the percentage inhibition, which was 62% (Table II). It shows that the product enhances the 34% activity of the enzyme or protects the enzyme from being inhibited.

Table II: Urease inhibition activities by thiourea in the absence and presence of product.

<table>
<thead>
<tr>
<th></th>
<th>Inhibition by thiourea in the absence of product (%)</th>
<th>Inhibition by thiourea in the presence of product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition by thiourea* in the absence of product</td>
<td>96</td>
<td>62</td>
</tr>
<tr>
<td>Inhibition by thiourea in the presence of product</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(* Standard)

Natural Orbital (NBO) analysis

NBO analysis is widely deployed recently to examine the bonds interaction and migration of charge densities from Lewis-type NBOs (filled or donor) to non-Lewis NBOs (vacant or acceptor) orbitals (Tahir et al., 2017; Adeel et al., 2017). Moreover, it is also believed that the NBO analysis is helpful for the detection of hydrogen bonding originates from hyper conjugative interactions. NBO analysis of investigated molecule was performed utilizing NBO 3.1 program which is an embedded option of Gaussian 09 package at B3LYP/6–311+G (d,p) level of theory and results are presented in Table III.

Table III: Second-order perturbation theory analysis of Fock matrix on NBO basis.

<table>
<thead>
<tr>
<th>Donor(i)</th>
<th>Type</th>
<th>Acceptor (j)</th>
<th>Type</th>
<th>E(2)</th>
<th>E(j)-E(i) [a.u.]</th>
<th>F(i; j) [a.u.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N4-N5</td>
<td>π</td>
<td>C17-C20</td>
<td>π*</td>
<td>10.14</td>
<td>0.40</td>
<td>0.061</td>
</tr>
<tr>
<td>C9-C12</td>
<td>π</td>
<td>O3-C14</td>
<td>π*</td>
<td>18.49</td>
<td>0.27</td>
<td>0.067</td>
</tr>
<tr>
<td>C9-C12</td>
<td>π</td>
<td>C21-C25</td>
<td>π*</td>
<td>19.77</td>
<td>0.29</td>
<td>0.069</td>
</tr>
<tr>
<td>C17-C20</td>
<td>π</td>
<td>N4-N5</td>
<td>π*</td>
<td>20.09</td>
<td>0.23</td>
<td>0.064</td>
</tr>
<tr>
<td>C17-C20</td>
<td>π</td>
<td>C19-C23</td>
<td>π*</td>
<td>20.95</td>
<td>0.27</td>
<td>0.068</td>
</tr>
<tr>
<td>C19-C23</td>
<td>π</td>
<td>C17-C20</td>
<td>π*</td>
<td>17.33</td>
<td>0.30</td>
<td>0.065</td>
</tr>
<tr>
<td>C21-C25</td>
<td>π</td>
<td>C31-C33</td>
<td>π*</td>
<td>21.72</td>
<td>0.28</td>
<td>0.070</td>
</tr>
<tr>
<td>C19-C23</td>
<td>π</td>
<td>C29-C35</td>
<td>π*</td>
<td>19.85</td>
<td>0.30</td>
<td>0.069</td>
</tr>
<tr>
<td>C29-C35</td>
<td>π</td>
<td>C17-C20</td>
<td>π*</td>
<td>20.78</td>
<td>0.28</td>
<td>0.069</td>
</tr>
<tr>
<td>C31-C33</td>
<td>π</td>
<td>C9-C12</td>
<td>π*</td>
<td>22.22</td>
<td>0.28</td>
<td>0.071</td>
</tr>
<tr>
<td>C9-C14</td>
<td>σ</td>
<td>C14-C27</td>
<td>σ*</td>
<td>0.57</td>
<td>1.06</td>
<td>0.022</td>
</tr>
<tr>
<td>N5</td>
<td>LP(1)</td>
<td>C17-C20</td>
<td>σ*</td>
<td>0.62</td>
<td>0.97</td>
<td>0.022</td>
</tr>
<tr>
<td>O3</td>
<td>LP(2)</td>
<td>C14-C27</td>
<td>σ*</td>
<td>20.13</td>
<td>0.67</td>
<td>0.105</td>
</tr>
</tbody>
</table>

To explore the donor-acceptor interactions, second order Fock matrix was carried out using equation (Snehalatha et al., 2009).

\[ E^{(2)} = q_i \left( \frac{F_{i,j}}{\varepsilon_j - \varepsilon_i} \right)^2 \]

In above equation, \( E^{(2)} \) represents the stabilization energy, \( q_i \) describes the donor-orbital occupancy, \( F_{i,j} \) points out the off diagonal NBO Fock matrix elements and \( \varepsilon_j \) and \( \varepsilon_i \) represents the diagonal elements. The most credible transitions takes place in our studied systems is \( \pi(C31-C33) \rightarrow \pi^*(C9-C12) \) with stabilization energy value 22.22 kJ/mol. This value is the largest one among all stabilization energy values presents in investigated molecule. On the other hand, \( \sigma(C9-C14) \rightarrow \sigma^*(C14-C27) \) transition is found to have least energy value 0.57 kJ/mol. This transition generates weak interaction between \( \sigma \) (donor) and \( \sigma^* \) (acceptor). Other transitions like \( \pi(C17-C20) \rightarrow \pi^*(C19-C23), \pi(C29-C35) \rightarrow \pi^*(C17-C20), \pi(C19-C23) \rightarrow \pi^*(C29-C35) \) and \( \pi(C9-C12) \rightarrow \pi^*(C21-C25) \) with stabilization energies 20.95, 20.78, 19.85 and 19.77 kJ/mol, respectively, represent the presence of conjugation in investigated molecule (Table III). In case of the resonance, the massive and least stabilization energy values 20.13 and 0.62 kJ/mol are observed to be for the transition LP(O3) \( \rightarrow \sigma^*(C14-C27) \) and LP(N5) \( \rightarrow \sigma^*(C17-C20) \) respectively. Above discussion confirmed the presence of extended conjugation in title molecules, hence, stabilization of the molecules due to intra-molecular hyper conjugative interactions.
Frontier Molecular Orbitals (FMOs)

HOMO and LUMO collectively form frontier molecular orbitals (FMOs). HOMO term is used to describe the highest occupied molecular orbital having higher energy, rich numbers of electron, therefore electron donating ability. Contrary, LUMO (lowest unoccupied molecular orbital) indicates the electron accepting capability due to lower energy and number deficiency of electrons. FMOs play a crucial role during molecular interactions. Furthermore, FMOs provide important perspective about the optical properties, electronic properties and reactivity of the molecule under investigation (Ebenezar et al., 2013; Sun et al., 2015). In this context, FMOs analysis were carried out at B3LYP (Becke, 3-parameter, Lee-Yang-Parr) level and 6311+G(d,p) basis set combination. Results of FMOs analysis are tabulated in Table IV. Four important molecular orbital pairs are examined and their pictographic display is presented in Fig., 5.

Table IV: Computed energy values for the title molecule in gas phase.

<table>
<thead>
<tr>
<th>MO(s)</th>
<th>$E$ (eV)</th>
<th>$\Delta E$ (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMO</td>
<td>-6.82</td>
<td>3.75</td>
</tr>
<tr>
<td>LUMO</td>
<td>-3.07</td>
<td></td>
</tr>
<tr>
<td>HOMO-1</td>
<td>-6.89</td>
<td>4.75</td>
</tr>
<tr>
<td>LUMO+1</td>
<td>-2.13</td>
<td></td>
</tr>
<tr>
<td>HOMO-2</td>
<td>-7.22</td>
<td>6.00</td>
</tr>
<tr>
<td>LUMO+2</td>
<td>-1.21</td>
<td></td>
</tr>
<tr>
<td>HOMO-3</td>
<td>-7.31</td>
<td>6.25</td>
</tr>
<tr>
<td>LUMO+3</td>
<td>-1.06</td>
<td></td>
</tr>
</tbody>
</table>

$E$= energy, $\Delta E$ (eV) = $E_{LUMO} - E_{HOMO}$
HOMO, LUMO are found to have energy values -6.82 and -3.07 eV respectively with an energy gap \(E_{\text{HOMO}} - E_{\text{LUMO}}\) value of 3.75 eV. Similarly, the calculated energy values of HOMO-1, LUMO+1 and \(E_{\text{HOMO}-1} - E_{\text{LUMO}+1}\) is found to be -6.89, -2.13 eV and 4.75 eV respectively. The energy value of HOMO-2, LUMO+2 and \(E_{\text{HOMO}-2} - E_{\text{LUMO}+2}\) is observed to be -7.22, -1.21 eV and 6.00 eV, respectively. In case of HOMO-3, LUMO+3 and \(E_{\text{HOMO}-3} - E_{\text{LUMO}+3}\), the calculated energy has been found around -7.31, -1.06 eV and 6.25 eV respectively (see Table IV and Fig., 5). The energies of HOMO, LUMO and their gap play a significant function in the prediction of global reactivity descriptors (Parthasarathi et al., 2004; Parthasarathi et al., 2004; Mahmood et al., 2015).

Global reactivity descriptors, such as electrophilicity index (\(\omega\)), electron affinity (\(EA\)), electronegativity (\(X\)), ionization potential (\(IP\)), global softness (\(S\)), global hardness (\(\eta\)) and chemical potential (\(\mu\)), are calculated using following equations and results are expressed in Table V.

From Table V, it is evident that the ionization potential value of title molecule is doubled than the electron affinity value which describes the better donating capability of the title molecule. Electron affinity value is found positive which depict that the investigated molecule might participate in charge transfer reactions. Electronegativity value of the title molecule is observed to be 4.952. The
The electrophilicity index of the compound is found to be 6.539. Global hardness value (1.875) of title molecule is found 7 times greater than their softness value (0.266). These findings suggest that the title molecule is a hard molecule with better donating capabilities.

**Table V**: Ionization potential ($I_P$), electron affinity ($E_A$), electro negativity ($X$) chemical potential ($\mu$) global hardness ($\eta$) global softness ($S$) and global electrophilicity ($\omega$).

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_P$ (eV)</td>
<td>6.827</td>
<td>6.89</td>
<td>7.22</td>
<td>7.319</td>
</tr>
<tr>
<td>$E_A$ (eV)</td>
<td>3.077</td>
<td>2.134</td>
<td>1.218</td>
<td>1.066</td>
</tr>
<tr>
<td>$X$ (eV)</td>
<td>4.952</td>
<td>4.512</td>
<td>4.219</td>
<td>4.192</td>
</tr>
<tr>
<td>$\mu$ (eV)</td>
<td>-4.952</td>
<td>-4.512</td>
<td>-4.219</td>
<td>-4.192</td>
</tr>
<tr>
<td>$\eta$ (eV)</td>
<td>1.875</td>
<td>2.378</td>
<td>3.001</td>
<td>3.126</td>
</tr>
<tr>
<td>$S$ (eV)</td>
<td>0.266</td>
<td>0.210</td>
<td>0.166</td>
<td>0.159</td>
</tr>
<tr>
<td>$\omega$ (eV)</td>
<td>6.539</td>
<td>4.280</td>
<td>2.965</td>
<td>2.810</td>
</tr>
</tbody>
</table>

A= HOMO & LUMO; B= HOMO-1 & LUMO+1; C= HOMO-2 & LUMO+2; D= HOMO-3 & LUMO+3

**CONCLUSION**

The product was obtained by the condensation of 3-chlorobenzophenone with acetylacetone with 87% yield. Its structure was elucidated by spectroscopic data. It showed reverse urease inhibition activity i.e., it enhanced the activity instead of inhibiting. NBO analysis showed that the interaction and migration of charge densities from filled to vacant orbitals in title molecule occurred and confirmed the presence of extended conjugation that leads to stabilization of the molecule. Also the findings of FMOs suggest that the title molecule is a hard molecule with better donating capabilities.

**ACKNOWLEDGMENT**

Dr. Muhammad Aslam expresses his compliments to HEC Islamabad, Pakistan for providing financial support (No. 21-193/SRGP/R&D/HEC/2014) and Department of Chemistry, University of Education, Township, Lahore for providing research facilities and also to Dr. Zahra Noreen, Dr. Abrar Hussain and Dr. Asma Chaudhary for providing facilities for biological activities.

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Understanding the community’s perception of climate change and adaptations in the Mid Hills of Pakistan

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ABSTRACT

Climate change poses a higher risk to the livelihood of vulnerable rural mountainous community due to prevailing poverty and more dependence on the natural resources of the surrounding areas. Aim of the study was to assess the local people’s perception of changing climate and to link it with meteorological data in the mid hills of Tehsil Balakot, KPK Pakistan. Using mix method approach of interviews and questionnaire, data was collected from 200 randomly selected households. Temperature data of 30 years 1985 to 2014 showed that there is an overall decrease in mean minimum annual temperature by a factor of 0.0024 for each year whereas there is an overall increase in mean maximum annual temperature by a factor of 0.0412 for each year. The mean annual rainfall of thirty years was recorded as 1471.27 mm with a decreasing trend of 0.9484 annually. Descriptive analysis showed that 83.5% and 40.5% of respondents observed change in temperature and increase in annual rainfall respectively. Local residents of the area were more vulnerable to climate change and natural hazards due to their poor socio-economic conditions and previous disasters history. In order to adapt to change in climatic conditions, mitigatory measures should be taken to improve their living conditions. Deforestation should also be avoided to overcome and reduce their vulnerabilities. Community resilience can help in mitigation and adaptation at local level.

Keywords: Climate change, Natural hazards, Mountainous Communities, Vulnerability

INTRODUCTION

Intergovernmental Panel on Climate change (IPCC, 2014) in its fifth assessment report state that the changing climate is one of the biggest challenges of the 21st century that will bring about unexpected extreme events to the whole world. In particular, South Asia is the home of one fifth of world’s population and is considered as most disaster prone region of the world (IPCC, 2007; Aryal et al., 2018). With ever increasing population coupled with poverty, natural resource dependence and degradation; this region is highly vulnerable to climate change and resulting natural disasters (Abid et al., 2016). Pakistan is among the most vulnerable and having higher economic losses due to natural disasters and climate change in South Asia (Akhtar et al., 2008, Shah et al., 2018). The additional burden of losses from natural disasters comes from the hazardous geographic location of the country (Ainuddin et al., 2013; Ullah et al., 2017). According to Risk management index calculated on Global scenario, Pakistan ranks in category 4 (2.0-2.4) which indicates poor disaster risk management and poor adaptive capacity with a high likelihood of having current and future disasters (Ali & Erenstein, 2016). United Nations Framework convention on Climate Change (UNFCCC, 2013) defines climate change as the weather pattern which departs from a decadal time span. The changing climate is even more evident in mountainous regions due to their marginalization, higher dependence on natural resources and extreme poverty (Macchi, 2011; Sarwar et al., 2016). Human activities linked with development have increased the concentration of greenhouse gases (GHGs) in the atmosphere thus warming it up (Maitib et al., 2017). The emission of GHGs has increased by 70% since the rapid
economic growth caused by the industrial revolution (Eggleston et al., 2006). Climate change is not just rising average global temperatures that concern scientists but also their effects on weather extremes, declining global ice cover and sea level rise. In fact, many of the predictions have made in the past about the impacts of global warming include disappearing glaciers, loss of sea ice, more extreme heat waves, accelerated sea level rise, and stronger hurricanes (Chaudhury et al., 2016; Choe et al., 2017). Adaptation enhances the capacity of people and governments to reduce climate change impacts (Akhtar et al., 2008). The term climate adaptation basically referred to the tendency to maintain as well as to improve the standards of living in such an appropriate manner that one could bear the severity and intensity of adverse climatic patterns (Aalst 2008).

Climate change is one of the most important global challenges affecting mountain ecosystems (Briner et al., 2013). Disasters hit mountainous communities and cause indirect great impacts downstream, affecting millions of people. Mountain climates vary noticeably with different exposures and provide limited resources. Inhabitants of these regions use their indigenous knowledge and developed sophisticated techniques for farming, livestock breeding, forestry and water use on steep slopes and in harsh unpredictable conditions (Wang et al., 2013). However, Hindu Kush Himalayan (HKH) is highly vulnerable to climate changes, affecting water volumes which directly affect the livelihood of locals (Elalem & Pal, 2013; Camp 2017).

The region is also at high-risk of avalanches, landslides, volcanic eruptions, earthquakes and glacial lake outburst floods which further threaten lives and livelihoods (Keating et al., 2016). As it can wipe out major livelihood resources such as standing crops, stored food, seeds, and fertile land (Veith, & Shaw, 2011), while fragile soils and vegetation cover make these areas more vulnerable to environmental degradation (Peterson & Halofsky 2017). Pakistan is alarmed to face future food insecurity majorly because of affected water availability due to changing climate. The water resources of HKH are life-line to Pakistan. Pakistan as an agricultural country has 47% population dependent upon agriculture with 24% contribution in national GDP (Ullah et al., 2015). With lesser contribution in global emission of GHGs, Pakistan suffers a lot from the consequences of climatic changes in terms of droughts, floods, weather alterations etc. (Akhtar et al., 2008; Ullah et al., 2017). This changing phenomenon is affecting the livelihood of people who have sufficiently less knowledge of adaptation to climate change. Better adaptation directly strengthens the resilience, which reveals the ability of systems or people to return to a former condition after facing stresses (IPCC, 2014). The local people have been using their traditional coping methods to sustain to changing environment but there is very little documentation of their techniques and accomplishments (Herman-Mercer et al., 2016).

Though climate change research has evolved from a bottom up approach rather than top-down approach, these people can be better instrumental to study the change analysis (Sonwa et al., 2016). Many studies have been carried out to assess farmer’s perception of adaptation to climate change throughout the world, there is still little work done in the mountainous regions of Pakistan. Keeping this research gap in consideration, the study therefore investigates perception of local people to assess climatic changes in Tehsil Balakot and how this change has affected the rural community and their livelihood; and what are their practices to mitigate the effects.

MATERIAL AND METHODS

Study area

Balakot (34° 33N; 73° 20E) is the biggest Tehsil of District Mansehra, KPK Province-Pakistan. It has an average elevation of 900m and one of the popular tourist stay over in the region (Soomro et al., 2010). The area was totally destroyed by the massive earthquake of 2005 and the losses were enormous ranging from death tolls to loss of agricultural land (UNDP, 2007). It is mostly mountainous and rural area with wage labor as major occupation, followed by agriculture and seasonal migration to main District Mansehra and Naran Valley. Majority of rural women were involved in livestock rearing and crop production. After agriculture, forest has become a major source of income in supporting livelihood (Soomro et al.,
Most of the community for their livelihood is totally dependent upon the ecological resources of the area, in the context of changing climate and dependence upon temperature and precipitation the community has become highly vulnerable in such marginalized region. Forest cover has changed over time due to use as fuelwood, the region is extremely cold winter nights and have no fuel available other than forest woods. Soil infertility, wind erosion and no irrigation infrastructure are the already documented reasons for the decrease in production of crops (Qasim et al., 2010). This scenario has provided our study a rational to know the perception of rural people and their mitigating strategies if any. The map of study area is shown in Fig. 1.

Fig. 1: Map of Tehsil Balakot indicating study sites

**Sampling procedure and study population**

Primary data for the study was collected from the field survey whereas secondary data was taken from many governmental departments including agricultural, irrigation and forest dept. Govt. of Pakistan. Tehsil Balakot has fifteen union councils (UCs), but population is unevenly distributed. Four villages from four UCs (Kawai, Mohandri, Balakot and Gari Habibullah) were chosen; the household survey of two hundreds houses was carried out chosen randomly to be the study population. Interviews were conducted using standard questionnaire applying qualitative and quantitative approach for data collection. In addition, Climatic data of temperature and precipitation was taken from the Balakot Station for a period of thirty years (1985 to 2014). Using mix method approach of data collection, interviews and house hold data was compared with climatic records of study area (Furberg et al., 2018).

**Field survey tool**

For primary data collection, a questionnaire was designed based on 34 items by thorough study of literatures which has helped in gathering information about demographic characteristics, social and economic conditions, and their perception regarding the climate change. A hazard mapping was carried out during a focus group discussion session after obtaining household data (Gentle and Maraseni 2012; Gentle et al., 2014).
Section 1: Socio-demographics of the locals
The first section inquired information about the respondents' demography. The information gathered was about their gender, age, and number of people living in their house. It also inquired about their education attainment, income of household, source of income, literacy rate, unemployment status and access to basic facilities like health facilities.

Section 2: Observation on climate change and adaptation
The section inquired about the perception impacts of climate change. Questions were asked to analyze the opinion of people about climate change and its impacts on their lives. This section was helpful in assessing the vulnerability of community to climate change and its relation with natural disasters. People’s perception about temperature change and its impacts on their income, variation in flowering and fruiting pattern, change in food production, change in food diversity, change in harvesting season, change in annual rainfall and its impact on productivity, change in glacier melting, snow pattern, biodiversity loss and availability of natural resources was inquired. This data helped us in developing a viewpoint of climatic changes in the region and how was the community adapting (Joshi et al., 2017).

Pilot study
The main purpose of conducting pilot study was to check the response of respondents towards the questionnaire and to check either the questions designed were understandable for posing correct idea. Twenty households were visited and information was collected as a part of pilot work. This helped in modification and improvement of study tool.

Main survey
Using the updated questionnaire, data was collected during a field survey interviewing two hundred head of the households; it was preferred that the head of household should be above 35 years of age and living in the area from last twenty years.

This was done to get a picture of changes observed in some time period (Shah et al., 2018).

Data analysis
The data collected was analyzed using SPSS version 21 to determine the frequency and percentage response, also regression trend analysis was done to assess change in climatic data. The regression analysis provided an

RESULTS AND DISCUSSION

Climatic trends of tehsil Balakot
The temperature records of over 30 years (1985-2014) collected from the Balakot meteorological station were mapped as mean annual minimum temperature and mean annual maximum temperature as shown in Fig., 2 and Fig., 3. Regression trend analysis showed that there was an overall decrease in mean minimum annual temperature by a factor of 0.0024 for each year whereas increase in mean maximum annual temperature was recorded by a factor of 0.0412 for each year.

An average analysis of temperature showed erratic trend of increasing and decreasing temperature throughout thirty years. But in case of minimum temperature an overall range of 11.2 °C to 14.1 °C was observed, minimum 11.2 was in year 2005 whereas 14.1 the highest among minimum temperatures was in year 2000. In terms of mean maximum temperatures a range of 22.6 °C was lowest in year 1997 with a highest value of 26.6 °C in year 2010 as shown in Fig., 3. This statistical trend of temperature was then compared with opinions of people during field survey. Most of them informed of their agriculture loss in year 2010 due to severe weather and resulting floods and landslides. The reported impacts were also linked with decreasing drinking water in springs and wells and loss in irrigation water. Similarly loss in forest cover, reduced availability of NTFP, and less grazing land was also reported by the locals.
Like temperature, there was unpredictable pattern of rainfall data which was taken from the Balakot Meteorological station as shown in Table- I. Overall the mean annual rainfall of thirty years was 1471.27 mm during 1985 to 2014. A drift in rainfall was calculated for a decadal period i.e. 1985-1994, 1995-2004 and 2005-2014 to observe changes over a time of period. From the data, a general trend was developed for the winter and summer rainfall which was similar according to the local people as well.

The monsoon period was considered as pre, post and mean monsoon months taking into account cropping seasons too. An increasing trend of mean winter rainfall was observed during 2005 to 2014; which was much higher than 1985-1994 and 1995-2004. This higher pattern of winter rainfall has influenced the wheat productivity as described by women during FGDs. Unpredicted rainfall was one of the identified hazard which impact community livelihood and ranked higher in hazard ranking as
shown in Fig., 3. The average rainfall in month of October and November was 46.8 and 36 mm respectively which was “minimum” recorded during thirty years span. A decreasing trend of rainfall was observed in months of March to May during 1995-2004 and 2005-2014. This was time when usually in past there was plenty of rainfall. There was a clear change in rainfall pattern for pre-monsoon period. It was also reported by the community and validated by the statistical data. Although the mean monsoon period was same for all three decades. Similar results of winter temperature and rainfall were produced by the work of Shah et al., 2010.

Table I: Pattern of Rainfall (mm) in Tehsil Balakot

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Annual (Jan-Dec)</th>
<th>Mean Winter (Dec-Feb)</th>
<th>Mean Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre-Monsoon (March- May)</td>
</tr>
<tr>
<td>1985-1994</td>
<td>1649.52</td>
<td>87.99</td>
<td>133.84</td>
</tr>
<tr>
<td>1995-2004</td>
<td>1348.48</td>
<td>67.93</td>
<td>99.14</td>
</tr>
<tr>
<td>2005-2014</td>
<td>1415.81</td>
<td>109.77</td>
<td>95.59</td>
</tr>
</tbody>
</table>

Source: Pakistan Meteorological Dept. (PMD) Govt. of Pakistan (2017)

Socio-demographic characteristic of surveyed population

An overall demographic picture of community was quantified and results are described in Table- II. In the first part gender of respondents were determined as male were 52% and female 48% respectively. Age of the respondents were asked, 69% of the respondents were between 21-30 years age group. The number of people living in a house was also determined to assess level of crowding. About 41.5% of people have spent 1-10 years. It was found that 68 % population had never visited the school or spent only primary levels at school. The number of people earning was also determined. Households containing 1-3 earning members were 75.5%. The source of income of the household was also determined. Almost all of respondents have opted agriculture as prime source, doing either as wage labor or cultivating for personal use. Livelihood diversification is considered as an adaptation strategy, it was seen that 78% of respondents either don’t know this or they had never thought of it which shows their poor adaptive capacity. Almost 76% people had affected land from previous disasters. In terms of energy, 61% households were relying on forest wood as a prime source for lightening, cooking, heating and other uses.

Table II: Characteristics of study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>N (%)</th>
<th>Variable</th>
<th>Description</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>104   (52)</td>
<td>Literacy level</td>
<td>Uneducated</td>
<td>136  (68)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>96    (48)</td>
<td></td>
<td>Educated</td>
<td>64    (32)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Less than 20 years</td>
<td>17    (8.5)</td>
<td>How many members in your</td>
<td>1-3 people</td>
<td>151 (75.5)</td>
</tr>
</tbody>
</table>
Perceptions on climate change and adaptations in mountainous region

An in-depth analysis was done to assess the observations of community for climate change and its adaptation. Percentage and frequency of questions inquired is presented in Table- III. Respondents were asked about their views about climate change and most of the respondents were familiar to term climate change whereas only 3.5% of the respondents were not familiar to term climate change. Reasons of climate change were also determined and 60.5% of the respondents indicated deforestation as a major reason of changing climatic conditions, whereas 6.5% of the respondents indicated pollution and energy exhaust, industrialization and global warming as reasons of climate change. Observation on the land use planning was resulted as 64.5% of the respondents said that there was no proper land use planning. In case of increase in disasters frequency and intensity, 59.5% of the respondents agreed a multitude. The change in temperature was also assessed. 83.5% of the respondents observed a change in temperature where 59.5% of the respondents said it has no effect on income.

Respondents were asked about the change in irrigation system and 34% of the respondents agreed of alteration. In terms of loss in biodiversity, 62.0% of the respondents related it with climate change. Change in flowering and fruiting pattern of crops was observed by 52% respondents, 41.5%
observed less food diversity and 40% observed change in harvesting season of crops. Change in annual rainfall was felt by 40% of the respondents whereas 43% also observed increase in glacier melting. Change in snow pattern of the area was also observed by 57.5% of the respondents however 71% of the respondents described loss in natural resources of their region after the earthquake of 2005 and floods of 2010 and 2013.

Table III: Observations of locals on climate change and adaptation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>N (%)</th>
<th>Variable</th>
<th>Description</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you know the term climate change?</td>
<td>Yes</td>
<td>193 (95.5)</td>
<td>Have you observed any change in temperature?</td>
<td>Yes</td>
<td>167 (83.5)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>7 (3.5)</td>
<td></td>
<td>No</td>
<td>14 (7)</td>
</tr>
<tr>
<td></td>
<td>No idea</td>
<td>0</td>
<td></td>
<td>No idea</td>
<td>19 (9.5)</td>
</tr>
<tr>
<td>What do you think are the reasons of climate change?</td>
<td>Deforestation</td>
<td>121 (60.5)</td>
<td>If yes, what is that change?</td>
<td>Increase in temp.</td>
<td>129 (64.5)</td>
</tr>
<tr>
<td></td>
<td>Land use change</td>
<td>66 (33)</td>
<td></td>
<td>Decrease in temp.</td>
<td>27 (13.5)</td>
</tr>
<tr>
<td></td>
<td>Any other</td>
<td>13 (6.5)</td>
<td></td>
<td>Both</td>
<td>13 (6.5)</td>
</tr>
<tr>
<td>Do you think there is proper land use planning in your region?</td>
<td>Yes</td>
<td>37 (18.5)</td>
<td>How these changes in temperature affect your income?</td>
<td>Increase in income</td>
<td>17 (8.5)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>129 (64.5)</td>
<td></td>
<td>Decrease in income</td>
<td>64 (32)</td>
</tr>
<tr>
<td></td>
<td>No idea</td>
<td>34 (17)</td>
<td></td>
<td>No effect</td>
<td>119 (59.5)</td>
</tr>
<tr>
<td>Do you agree that earthquakes are regular feature of your area?</td>
<td>Yes</td>
<td>118 (59)</td>
<td>Is this change in climatic conditions affecting your health?</td>
<td>Yes</td>
<td>143 (71.5)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>82 (41)</td>
<td></td>
<td>No</td>
<td>46 (23)</td>
</tr>
<tr>
<td>Does the disaster increased here?</td>
<td>Yes</td>
<td>119 (59.5)</td>
<td>Have you observed any change in flowering and fruiting pattern of crops?</td>
<td>Yes</td>
<td>104 (52)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>81 (40.5)</td>
<td></td>
<td>No</td>
<td>53 (26.5)</td>
</tr>
<tr>
<td></td>
<td>No idea</td>
<td>11 (5.5)</td>
<td></td>
<td>No idea</td>
<td>43 (21.5)</td>
</tr>
<tr>
<td>Question</td>
<td>Yes</td>
<td>No</td>
<td>No idea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>-----</td>
<td>--------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you understand term resistance to be applied in your cropping system?</td>
<td>39</td>
<td>97</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Which crop is abundant here?</td>
<td>34</td>
<td>65</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are the natural resources depleted after the disaster?</td>
<td>142</td>
<td>31</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do the people living here are suffering from more health problems?</td>
<td>166</td>
<td>28</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is the climate change leading to biodiversity loss?</td>
<td>102</td>
<td>84</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you think the organizations are considering climate change an issue?</td>
<td>124</td>
<td>25</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is there any change in food diversity over past ten years?</td>
<td>83</td>
<td>52</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is there any change in harvesting season of crops?</td>
<td>Yes</td>
<td>No</td>
<td>No idea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you observed any change in annual rainfall?</td>
<td>Yes</td>
<td>No</td>
<td>No idea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you felt any change in glacier melting?</td>
<td>Yes</td>
<td>No</td>
<td>No idea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>If yes, did it result in formation of artificial lake?</td>
<td>Yes</td>
<td>No</td>
<td>No idea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is there any reservoir to store flood water?</td>
<td>Yes</td>
<td>No</td>
<td>No idea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less diversity</td>
<td>19</td>
<td>86</td>
<td>62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>More diversity</td>
<td>14</td>
<td>48</td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>no idea</td>
<td>52</td>
<td>85</td>
<td>32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Lastly people were asked to describe the hazards/disasters to which they had higher exposure in the period of last ten years and their response was mapped through the hazard ranking system of 0 to 5. Here 0 indicates least and 5 as highest level of exposure. It showed that community felt higher hazard of earthquake with a rank of 5; landslides, floods and rainfall were at 4th rank. The reason was the deadliest earthquake of 2005 from which they still couldn’t recover loses. In addition to this, landslides were common phenomenon, and change in rainfall pattern had adversely affected their livelihood.

Overall, perception of the local people about the climate change determined that they were aware of the climate change in their region. Increase in temperature, effect of temperature increase on their health, change in annual rainfall, change in snow pattern and glacier melting were the common perception of the local community about climate change. A case study by Tse-ring et al., (2010) revealed that change in temperature in a region affects the precipitation rate directly but indirectly it affects the health of the local people. The data collected from Tehsil Balakot concluded that the change in temperature in the area over past
15 to 20 years was affecting the health of local residents at high pace. People were facing high temperature, and infected by the viral diseases on regular basis. Similarly, relation between harvesting season and the crop abundance revealed that due to variation in climatic conditions, harvesting season was shifted which was affecting the yield and the abundance of crop in the area. Our results were similar to the findings of Chaturvedi et al., 2011.

A case study conducted by Chaudhary and Bawa (2011) revealed that over all temperature is increasing with less annual snowfall, glacier retreat, and drying up of water resources. These results were alike to the outcomes of our study. It was assessed that no disaster resistant crops were introduced in the area as an adaptive measure taken to reduce the vulnerability to climate variability; the findings are similar to the study of Alam et al., 2017. Local residents observed change in annual rainfall over the past 15-20 years. People were of the opinion that they received heavy rainfall as compared to past years and the heavy rainfall has direct impact on their agriculture activities. Our results were similar to that of study conducted in KPK by Abid et al., 2016.

Overall it was identified the people with better income and education were in better position to mitigate the changing climate by having livelihood preferences (Shah et al., 2018). Most of households reported change in their agriculture productivity and problems regarding cropping due to water availability and changing temperature. All of them mentioned about reduced forest cover (Qasim et al. 2010) and difficulty in getting fuel wood as it was recently banned by the local government. The poor households had difficulties with their day-to-day livelihood activities; similar results were presented by Gentle and Maraseni (2012) in their work in Jumla District of Nepal which is also a mountainous region. Most of the community responses’ were significant with records of temperature and precipitation.

**CONCLUSION**

Using a participatory data technique in Tehsil Balakot, experiences of local people were noted which were supported by the meteorological data that climate change is happening in the area and influencing their livelihood negatively. The local people were key factors in mapping and identifying community hazards. Climatic data was taken to validate the perceptions of people, which proved to be an effective tool as viewpoint highlighted during interviewing was matching with the records of temperature and precipitation. In the study area ecological resources were degraded due to increasing population pressure and change in livelihood patterns. The daily life of locals was altered because of change in rainfall and temperature which further impacted their agricultural productivity, change in seasons, reduced water volumes etc. People were not prepared for the long term changes in their weather patterns which will change their day to day livelihood. The study has concluded that such prone areas need special attention from the policy makers to help the local community from natural hazards and adaptation.

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Structure prediction of ORF3 encoded protein of a novel Pakistani avian hepatitis E virus strain

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ABSTRACT
Avian HEV (aHEV) has been considered as causative agent of hepatitis-splenomegaly syndrome (HSS) in chickens. Like other HEV strains, it is single-stranded positive sense RNA virus belonging to Hepeviridae. In the present study we report partial structural homology of ORF3 encoded protein (ORF3P) of Pakistani novel aHEV (Pak naHEV) strain with a kinase 2-amino-4-hydroxy-6-hydroxymethylidihydropyridine pyrophosphokinase (HPPK) which participates in folate biosynthesis in bacteria. This finding is in agreement with the role of ORF3P as multifunctional protein modulating host cell signaling and gene expression to promote viral replication during infection.

Keywords: Avian HEV, ORF3, Kinase, Pakistan

INTRODUCTION
Avian HEV, like other HEV strains, is single stranded-positive RNA virus which belongs to genus Orthohepevirus, which is placed in family Hepeviridae as a separate species Orthohepevirus B (Smith et al., 2014; Smith et al., 2015; Purdy et al., 2017). The length of aHEV genome is approximately 6.6 kb which is 600 bp shorter than those of mammalian HEV (mHEV) genotypes. The shorter portion has a putative missing region between MeT and Hel (Reuter et al., 2016a). The genome organization is almost same as mHEV with three open reading frames (ORFs); ORF1, ORF2 and ORF3. ORF1 encodes single non-structural polyprotein, with multiple functional domains. ORF2 encodes capsid protein, while ORF3 codes for a small multifunctional regulatory protein (Huang et al., 2004).

The aHEV is associated with HSS and Big Liver and Spleen (BLS) disease in chickens (Haqshenas et al., 2001; Huang et al., 2002; Marek et al., 2010; Payne et al., 1999). Decreased egg production, high mortality rate, hepatitis and splenomegaly have been identified as common clinical symptoms of this infection (Jhone et al., 2014). At present, worldwide four different genotypes (Gt) of aHEV isolated from chickens have been reported. In Australia, United States of America (USA), China and Taiwan only a single genotype (Gt1, Gt2, Gt3 and Gt4, respectively) is circulating in chicken population but in Korea (Gt1, Gt2) and Hungary (Gt3, Gt4) two different genotypes are co-circulating among chickens (Zhao et al., 2015; Moon et al., 2016). Prevalence of HEV in other bird species has been reported in many studies. A novel avian-like HEV was reported in wild little egret (Egretta garzetta) (Reuter et al., 2016a). But interestingly in carnivorous wild prey bird kestrel (Falco tinnunculus) and red-footed falcon (F. vespertinus) novel HEV strains related to mammalian HEV (mHEV) from ferret and rat, were identified (Reuter et al., 2016b).

The ORF3P is a multifunctional protein which is transcribed from bicistronic subgenomic RNA (Graff et al., 2006). Its overall structure presented to have hydrophobic domains towards the N-terminal and proline-rich domain (s) (PRD) in C-terminal (Kannan et al., 2009; Holla et al., 2013). A single PRD (PREPSAPP) with a PSAP motif has been identified in aHEV ORF3P. This PRD is known to be a binding site for SH3-domain proteins (SDP) and vaculor sorting proteins (VSP). The interaction of PSAP motif with components of...
endosomal sorting complex (ESC) and host tumor suppressor gene 101 (TSG101) suggests role of ORF3P in triggering release of enveloped virions from cellular membrane of host cell (Jouvenet et al., 2011; Kenney et al., 2012; Nan et al., 2014, 2015). The role of ORF3P as a viroporin is elaborated in a recent study which facilitates virus egress from host cell (Jouvenet et al., 2011; Kenney et al., 2012; Nan et al., 2014, 2015). The other important function of ORF3P is modulation of host cell signaling and genes expression; for instance MAP kinase activation, down regulation STAT3 mediated genes, inhibition of mitochondrial apoptosis pathway and interferon-α mediated signaling inhibition (Kar-Roy et al., 2004; Chandra et al., 2010; Chandra et al., 2011; Dong et al., 2012). In a recent study a wide range of host proteins were found to which ORF3P interact in combination of other HEV proteins (Subramani et al., 2018).

The role of ORF3P in modulation of host cell signaling and gene expression may build an assumption of its function as a kinase and may be participating, directly or indirectly, in host proteins phosphorylation which is an important regulatory mechanism (Ardito et al., 2017). In connection to this in the present study our data based on ORF3P structure prediction and modeling analysis of Pak naHEV strain revealed homology with 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase (HPPK) which is an enzyme of folate biosynthetic pathway (Yang et al., 2005). The potential function of ORF3P as a kinase needs further investigation.

**MATERIALS AND METHODS**

**Sampling**

The samples of liver tissue, bile fluid and feces were collected from 19 layer chickens (dead before less than 12 hours) aged 30 – 90 weeks, from different poultry forms situated in Pattoki Punjab, Pakistan (31° 1' 0" North, 73° 51' 0" East). The mortality was not simultaneous and no outbreak was reported in the area. The sterilized 1.5 ml microfuge tubes were used of bile fluid and fecal samples collection, transported to Molecular Biology lab of Department of Zoology, University of Gujrat on ice packets and stored at -80°C in ultralow freezer for future analyses. The bile and fecal samples were designated as PT1B – PT19B and PT1F – PT19F, respectively.

**Detection of Viral RNA and Complete ORF3 Amplification**

Total RNA was isolated from bile and fecal suspension through Trizol (Invitrogen) method following manufacturer instruction. The cDNA synthesis was done through Reverse Transcription PCR (RT-PCR), using both gene specific primers as well as random hexamer primers. For random hexamer-primers VILO 2X master mix (Invitrogen) was used. To detect viral RNA in samples, partial helicase (ORF1) 186 bp and partial capsid protein (ORF2) 280 bp fragments were amplified (Kwon et al., 2012) using PerfeCta SYBR Green FastMix 2X (Quantab Biosciences). The primer set for helicase used; forward (RPHF) 5’-TGCCGACAY-GTWTCYCACCG–3’ and reverse (RPHR) 5’-CCTCRTGGAGCCTWATCGACC-3’ and primer set for capsid protein used was; forward (RPO2F) 5’-GGTATGGTTGATTTTGCCATAAAG-3’ and reverse (RPO2R) 5’-GCTGCNCGNARCAGTG-TCGA-3’. The PCR was carried at; initial denaturation 95 °C for 2 min followed by 40 cycles of 95 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min and final extension 72 °C for 10 min. PerfeCta SYBR Green FastMix 2X (Quanta Biosciences) was used for amplification of complete ORF3 using primer set; forward (APO31S) 5’-ACCATCC-AGCTTGTTGGCCG–3’ and reverse (APO31A) 5’-CACAAACCATGAGCATGCGGGACC-3’. The PCR conditions followed were; initial denaturation 95 °C for 2 min followed by 40 cycles of 95 °C for 45 sec, 55 °C for 45 sec, 72 °C for 3 min and final extension 72 °C for 10 min.

**Sequence analysis and phylogeny**

The obtained sequences were compiled through DNASTAR software (Lasergene). Then these sequences were used for homology analysis through NCBI Basic Local Alignment Search Tool (BLAST) (Zhang et al., 2000; Morgulis et al., 2008). Further, percent identity (PI) multiple sequence alignment (MSA) and phylogenetic analysis was done by MEGA 6 (Tamura et al., 2013) and ClustalX2.1 (Larkin et al., 2007) by comparing with other aHEV and mHEV strains.

**Protein structure prediction and modeling**

Protein structure prediction and modeling was carried out through SWISS-MODEL (Biasini et al., 2014). The deduced amino acids sequence was
used as input data and templates from SWISS-MODEL Template Library (SMTL) were obtained through BLAST and HHBlits and the highest quality templates were selected. On the basis of template data, structure modeling was done using ProMod3 (Guex et al., 2009) and model quality, at global and per-residue level, was evaluated by QMEAN scoring function (Benkert et al., 2008; 2011). Moreover, the quaternary structure of the model in its oligomeric form was estimated according to Bertoni et al. (2017).

**Complete ORF3 cloning**

The complete ORF3, along with flanking regions from ORF1 and ORF2, was cloned through TOPO TA cloning kit (Invitrogen) in TOPO XL pCR cloning vector following manufacturer instructions.

**RESULTS**

Two novel strains of Pakistani aHEV (PT12B, PT16B) were isolated from the bile fluid of two layer chickens aged 70 weeks. Sequence analysis on the basis of ORF3 revealed that they shared 100% identity which suggests that it is a single strain circulating in layer chickens population in the area.

**Molecular detection of Pak naHEV**

The bile and fecal samples collected from layer chickens were processed for molecular detection through PCR. No fecal sample was found positive while only two (10.5%) bile samples, PT12B and PT16B, were found positive for aHEV RNA (Fig. 1).

**ORF3 amplification and sequence analysis**

A fragment of 902 bp was amplified from aHEV RNA positive bile samples PT12B and PT16B (Fig. 2). This fragment contained complete ORF3 (264 bp) with some portion of ORF1 at 5’ end and 3’ end. So, it represents typically the junction of three ORFs. Complete nucleotides sequence of 902 bp fragment was obtained from TA clones by sequencing using vectors primers (M13). Sequences were submitted to GenBank under accession numbers MH018052 and MH018053.

Table I presents summary of sequence identity analysis of aHEV strains, including Pak naHEV strains, with mHEV strains from different genotypes showing the highest sequence identity (95%) with sequences AM943647 and EF206691. It was observed that all aHEV strains analyzed, including Pak naHEV, showed highest sequence identity (45%, 46%) with mHEV strains from Gt4 (FJ763142, AJ272108). Restriction analysis of complete ORF3 nucleotides sequence of Pak naHEV and other aHEV strains for commonly used restriction enzymes, demonstrated a conserved restriction site GGTACC of Kpnl at 255 nt position in all strain (data not shown). Multiple sequence alignment (MSA) of Pak naHEV strains complete ORF3 deduced amino acids sequence with other aHEV strains identified conserved amino acids sequence motifs at positions; aa 1-12, aa 16-28, aa 36-44, aa 55-58, aa 60-64, aa 66-72 and aa 76-87 (Fig. 3). Distant clustering of Pak naHEV strains was shown as result of phylogenetic analysis based on complete ORF3 deduced amino acids sequence with other aHEV strains (Fig. 4).

Comparison of complete ORF3 deduced amino acids sequence of aHEV strains, including Pak naHEV, with mHEV strains identified sequence gaps in aHEV ORF3 at following positions (reference AAA45726); 1-10 aa, 25-28 aa, 52-54 aa, 60-63 aa, 87-94 aa and 108 aa. Similarly, some conserved amino acids were also identified at positions (according to AM943647); C(9), L(10), C(12), G(33), G(36), P(38), Q(42), P(43), P(78) and R(84) (data not shown).

**ORF3 protein structure and modeling**

As both Pak naHEV strains showed 100% sequence identity (nucleotides and amino acids) of complete ORF3 with each other, so the identical structure models were obtained on the basis of complete ORF3 deduced amino acids sequence (Fig. 5,6) The Pak naHEV ORF3P structure model built with ProMod3 on the basis of homology mining showed that C-terminal region, from aa 47-86, is structurally homologous to a kinase HPPK (aa 35-74) (1rtz.1.A) (Fig. 5, 6 and Table II) . The Pak naHEV ORF3P model presented 3 β-sheets followed by single α-helix at the extreme C-terminus and loop structures connect β-sheets and α-helix with each other. The superimposition of Pak naHEV ORF3P model on its template homologous region is also shown (Fig. 5B, 6d). Fig. 6 demonstrates different structural parameters of Pak naHEV ORF3P structure model. The comparison with non-redundant PDB structures identified that over all Z-score of the model is less than 2 and greater than 1 which suggests good quality of the model (Fig. 6).
DISCUSSION

The Pak naHEV strains shared 100% sequences identity of complete ORF3 with each other which suggests that a single novel aHEV is circulating in the layer chickens population of the area. The overall sequence identity range presented was 93 – 95% for other aHEV strains and 41 – 45% for mHEV strains of different genotypes. The above mentioned sequence identity range is almost in agreement with earlier studies (Haqshenas et al., 2001; Huang et al., 2002). At present only 4 aHEV genotypes are reported (Zhao et al., 2015) but phylogenetic analysis based on ORF3 deduced amino acids sequence presented distant clustering of Pak naHEV which suggests that it belongs to a previously unknown genotype.

The role of ORF3P is as multifunctional protein is quite evident and it plays with host cell signaling and gene expression during course of infection (Ding et al., 2017; Nan et al., 2016). Our data showed that the C-terminal (aa 46-86) of ORF3 is structural homology to 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase (HPPK) which is a kinase and plays important role in folate biosynthesis.

The corresponding homologous portion of HPPK (aa 35 – 74) represents loop 2 and adjacent area which is a component of active site. The active site of HPPK is palm like structure in which loop 2 and 3 are flexible and used for ligand recognition and probably catalysis (Yang et al., 2005; Pemble et al., 2010). These findings may reinforce role ORF3P to interact host cellular signaling and hence modulation of host immune responses and other functions which promote viral replication (Tong et al., 2016). The function of ORF3P as a kinase to phosphorylate host proteins for cell signaling and host gene expression modulation is not reported so far. Moreover, the ORF3P homologous region represents only a part of HPPK active site, so further investigation is needed in this regard.

ACKNOWLEDGEMENT

Avian HEV (aHEV) positive control samples (fecal) were kindly provided by X.J. Meng, Center for Molecular Medicine and Infectious Diseases, Virginia Tech, VA USA.

REFERENCES


Table I: Pak naHEV strains complete ORF3 nucleotides sequence based PI Matrix with other aHEV and mHEV strains

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Over all PI of Pak naHEV strains with each other = 100%, with other aHEV strains = 93 – 95%, with mHEV strains = 41 – 45%. Highest PI in bold
Table II: Pak naHEV strain ORF3P homology modeling template information

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| 1rt7.1.A | 27.50        | monomer     | HHblits  | X-ray  | 1.33Å      | 0.31           | 47 - 86 | 0.46     | 2-amino-4-hydroxy-6-hydroxymethyl dihydroptero
dine pyrophosphokinase |

Fig. 1: Detection of aHEV genome in bile and fecal suspension samples of PT12 and PT16 chicken (0.8% Agarose Gel). Lane 2 – 7 helicase (186 bp, arrow head) and lane 8 – 13 ORF2 amplification (280 bp, arrow head); lane 2 = PT12B, lane 3 = PT12F, lane 4 = PT16B, lane 5 = PT16F, lane 6 = positive control, lane 7 = negative control, lane 8 = PT12B, lane 9 = PT12F, lane 10 = PT16B, lane 11 = PT16F, lane 12 = positive control, lane 13 = negative control, lane 14 = λ-Hind III DNA marker, lane 1 = λ-Hind III DNA marker (B = bile sample, F = fecal sample)

Fig. 2: Amplification of complete ORF3 (902 bp, arrow head) from Pak naHEV strains bile samples (2% Agarose Gel). Lane 1 = PT12B, lane 2 = PT14B, lane 3 = PT16B, lane 4 = positive control, lane 5 = negative control, lane 6 = 1 kb plus DNA marker

Fig. 3: Multiple sequence alignment of complete ORF3 deduced amino acids sequence of Pak naHEV strains (MH018052, MH018053) with other aHEV strains.
Fig. 4: Phylogenetic relationship of Pak naHEV with other aHEV strains on the basis of complete ORF3 deduced amino acids sequence. Asterisk shows genotype 1 strains which are distantly clustered in this case. Numbers shows branch length.

Fig. 5: Pak naHEV strain ORF3P homology modeling. A) alignment with template structure (1rtz.1.A), B) structure model showing identical (in blue colour) portion in template structure (1rtz.1.A)
Fig. 6: Pak naHEV strain ORF3P structure model, a) Z-score of different structure parameters, b) local quality estimate of each amino residue, c) comparison of built model with non-redundant set of PDB structures, d) final Pak naHEV ORF3P model


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Estimation of antibacterial action of *Aloe Vera* L. on different strains and concentrations

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ARTICLE INFORMATION

ABSTRACT

Aloe vera L. belonging to family Liliaceae is a very important worldwide used plant having nutritional as well as medicinal value. It plays an important role in antimicrobial action. The current study was aimed to assess of antibacterial activity of ethanolic, methanolic and aqueous extracts of *Aloe vera* L. against six bacterial strains (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Shigella sonnei*, *Escherichia coli* (1) (ATCC 13048) and *Neisseria gonorrhoeae*). Agar well diffusion method was applied to assess the antibacterial activity of these extracts. Their antibacterial activities were associated with typical chemical like gentamicine (antibiotic) as positive control. This study revealed that *S. pyogenes*, *S. aureus* and *N. gonorrhoeae* had the maximum inhibition to different extracts of *A. vera* plant and selected plant extracts remained less effective against *Shigella sonnei*, *E. coli* (1) and *E. coli* (2). The data also revealed that methanolic extract showed relatively more inhibitory effects on bacterial growth as compared to other solvent extracts.

Keywords: *Aloe vera*, Antibacterial activity, Aqueous solution, Ethanolic extract, Methanolic extract

INTRODUCTION

Plants have been utilized as valuable source of medicine and food from the ancient times. Numerous plant extracts have constituents with anti-infectious activity. People utilized enormous quantities of plants for the medicinal purposes (Rahmatullah *et al.*, 2009). Many traditional medicines are prepared by using these plants still used in rural areas of many developing countries. These herbal medicines are relatively safe and cheaper than synthetic medicines (Moshiuzzaman *et al.*, 2008). Therefore, herbal medicines are valuable source of treating disease for people living in rural community. *Aloe vera* L. is a perennial fleshy xerophyte, which stores water reserve in the leave tissue to survive in dehydrated regions of little and unpredictable rainfall. Internal chunk of the leaf is moist, soft, glossy, pulpy and lubricious which contains numerous thin-walled parenchyma tissues where water is available in the shape of gummy mucilage (Araújo *et al.*, 2013). Its cell wall comprises of cellulose and hemicelluloses as well as accumulated carbohydrates such as acetylated mannann. Many people use gel of Aloe as a therapy against skin infections such as sunburn, heat burns, psoriasis, cold sore and frostbite (Mushtaq *et al.*, 2012). It is also used to treat bowel diseases, osteoarthritis, fever, body inflammation and itching. It is also used for stomach ulcers, diabetes and asthma intended for soothing side-effects of radio therapy. It is helpful in natural treatment of constipation and depression (Haq, 2004). *A. vera* has many medicinal uses and from the ancient time has been used to treat different diseases like, genital herpes, epilepsy and dandruff (Qadir, 2009). When used as a mouth rinse, pure juice of Aloe is very effective at reducing dental plaque build-up as regular mouthwash and also aid in the recovery of mouth ulcers (Eamlamnam *et al.*, 2006). It is also used as natural healing agent for many diseases of skin. It was observed that there were special properties of *A. vera* extract with respect to different organisms specially human...
being (Channa et al., 2014). However, due to curative and therapeutic properties Aloe has been used for many decades containing numerous active ingredients in its gel, out of them over 75 have been recognized (Rice-Evans et al., 2004; Hamman, 2008). Polysaccharides are present in the inner parenchyma tissues of pulp of Aloe leaf are used as medicines (Purohit et al., 2012).

A group of compounds has been reported in A. vera L. leaf that perform biological activities especially in cancer (Habeeb et al., 2007; Masaldan et al., 2011). It has more than thirty active components used in cosmetics as well as in medicines production. Aloe contains about 98% water. Water removed from those active constituents by applying reverse osmosis system and save those active constituents from thermal damage of active compounds present in the juice (Abdullah et al., 2014). It has been reported that some essential heavy metals like Nickel (Ni), Chromium (Cr), Cobalt (Co), Iron (Fe), Copper (Cu) and Zinc (Zn) are present in A. vera. Besides them some of the non-essential and toxic metals like, Cadmium (Cd) and Lead (Pb) were also found in Aloe leaves (Iqbal et al., 2013). Its juice comprises of pulp containing natural ingredients and fibres. As herbal medication for the skin it also has high role in internal healing, cleansing and refurbishing. People prefer plant based medicines than synthetic medicines and Himalaya Agri herbal Aloe products are in high demanding in the international markets such as Asia, USA, Australia and whole Europe (Cristiano et al., 2016). Aloe species provide commercial platform and extraction of its pulp has been done in industries at vast scale around the globe. Therefore, the current study was planned to evaluate antibacterial activity of A. vera L.

MATERIALS AND METHODS

To determine the antibacterial activity of Aloe vera L. experiments were carried out in Ethnobotany Laboratory of the Department of Botany, University of Gujrat (UOG), Punjab, Pakistan.

Identification and collection herb

Aloe vera L. plants were purchased from local nursery of Gujrat, Punjab, Pakistan, and identified with the aid of Flora of Pakistan (Freitag, 2001)

The plant leaves were washed with distilled water to remove dirt and after that these were air-dried for 2 weeks at 37°C. Then, finely crushed to form decent uniform paste by means of crusher and kept in dark in air tight vessels (Sandasi et al., 2010).

Extraction of plant materials

Based on polarity, gel-like Aloe leaves samples were thoroughly mixed to prepare ethanolic, methanolic and aqueous extracts. Plant samples (10 g) were mixed with 100 ml solvents and placed these solutions for 24 hours at 190-220 rpm on incubator shaker. Thereafter, the solvents were filtered with Whatman filter paper and poured in to the falcon tubes. Methanolic and ethanol extract were placed in a rotatory evaporator at 60 and 40 °C at 250 rpm to evaporate 60% of the solvent to get crude extract. After that, the remaining 40% solvents were vaporized in an incubator. The extracted material was kept in a refrigerator at 18 ºC for further use. The crude extract was further mixed in 1% of Dimethyl Sulfoxide (DMSO) solution to formulate various concentrations as described by Alipour et al. (2011).

Culturing of bacterial strains

Six bacterial strains were obtained from the Biochemistry Department Lab. University of Gujrat. These bacterial strains were identified using biochemical techniques and further sub-cultured on Luira Broth (LB) Medium for 18-24 hours at 35-37 °C (Shameem et al., 2017).

Table 1: Pre-Cultured Bacterial strains used for antibacterial assay

<table>
<thead>
<tr>
<th>Sr. #</th>
<th>Bacterial Strains</th>
<th>Accession No.</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td><em>Streptococcus pyogenes</em></td>
<td>ATCC 15224</td>
</tr>
<tr>
<td>2.</td>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 25922</td>
</tr>
<tr>
<td>3.</td>
<td><em>Shigella sonnei</em></td>
<td>ATCC 35318</td>
</tr>
<tr>
<td>4.</td>
<td><em>Escherichia coli 1</em></td>
<td>4C11</td>
</tr>
<tr>
<td>5.</td>
<td><em>Escherichia coli 2</em></td>
<td>ATCC 13048</td>
</tr>
<tr>
<td>6.</td>
<td><em>Neisseria gonorrhoeae</em></td>
<td>TC-11-2</td>
</tr>
</tbody>
</table>
**Agar well diffusion assay**

To calculate the antibacterial activity of Aloe extracts, agar well diffusion method was used with some modifications (Rana et al., 2011). The bacterial strains were incubated on LB medium and after 12-18 hrs, spread with sterilized spreader on agar containing petri plates. With the help of Glass borer about 6mm wells were developed in the agar in the Petri-plates. Crude extracts of Aloe prepared in 1 % DMSO were poured into wells to evaluate antibacterial activity. All the three extracts of Aloe vera in Methanol, water and Ethanol were used for the said purpose. Six clinical strains of bacteria (Table 1) S. pyogenes, S. aureus, S. sonnei, E. coli 1, E. coli 2 and N. gonorrhoeae were used to investigate the activity of Aloe extracts. Three concentrations (25, 50 and 75 mg/ml) of plant extract residues in DMSO were used to investigate of the lowermost effective concentration of Aloe.

DMSO was used as a negative control in this experiment. At the same time, standard antibiotic (control) was added as positive control in adjacent well and inhibition zones (IZ) diameter was calculated for each extract well. The experiments were set up with three replicates. The readings were noted, their means were determined for determining the antibacterial activity of plant extracts (Saleem et al., 2010). The corresponding inhibition zone diameter was measured after 24 hours and 48 hours, respectively.

**RESULTS**

**Antibacterial action of aqueous extract of Aloe vera after 24 and 48 hours**

Aqueous extract of Aloe at conc. (25 mg/100, 50 mg/100 and 75 mg/100 ml) in DMSO solvent showed generally decline in bacterial growth after 24 and 48 hours of incubation. *Escherichia coli* (1) (4C11) and *Escherichia coli* (2) (ATCC 13048) were reported to be sluggish at 25 mg/ml treatment of aqueous Aloe extract but, they showed some inhibition in growth at 50 mg and 75 mg of plant extract. However, all bacterial strains showed variable inhibition zones at different conc. of aqueous extract. *Streptococcus pyogenes* indicated significantly high antibacterial activity while, *Escherichia coli* (1) showed least antibacterial activity in aqueous extract after 24 hours (Fig. 1a) and 48 hours (Fig. 1b) of incubation. The antibacterial activity of Aloe was comparable with standard antibacterial agent i.e., Gentamicin of Pfizer company.

**Antibacterial action of methanolic extracts of Aloe vera after 24 and 48 hours**

Methanolic extracts of Aloe in DMSO indicated the effective inhibition bacteria strains after 24 and 48 hours incubation at conc. (25 mg/ml, 50 mg/ml and 75 mg/ml). All bacterial strains showed maximum IZ at 75 mg/ml conc. methanolic extract. However, S. aureus and S. pyogenes exhibited the maximum IZ as compared to E. coli 1 and E. coli 2 after 24 hours (Fig. 2a) and 48 hours (Fig. 2b) of incubation.
Fig. 2a: Antibacterial action of methanolic extract of A. vera after 24 hours of incubation

Fig. 2b: Antibacterial action of methanolic extract of A. vera after 28 hours of incubation

Antibacterial action of ethanolic extract of Aloe vera after 24 and 48 hours

The ethanolic extract of A. vera at various conc. in DMSO exhibited the remarkable reduction in bacterial strains growth after 24 hrs and 48 hrs incubation at conc. (25, 50 and 75 mg/ml). However, S. aureus showed maximum inhibition as compared to E. coli 2. The ethanolic extract of Aloe displayed maximum antimicrobial activity against S. aureus and minimum against E. coli 2 after 24 hrs (Fig. 3a) and 48 hrs (Fig. 3b) of incubation.

Fig. 3a: Antibacterial action of Aloe vera extract in ethanol after 24 hours of incubation.

Fig. 3b: Antibacterial action Aloe vera extract in ethanol after 48 hours of incubation.

DISCUSSIONS

The data showed that Aloe vera had a great potential of controlling growth different bacterial strains. Methanolic, ethanolic and aqueous extracts of Aloe caused inhibition in bacterial growth. Our results are coherent with the findings that Psidium guajava extracts exhibit antimicrobial effects against both gram positive and negative bacteria. The antibacterial activity of Aloe might be owing to the prevalence of the bioactive polyphenolic elements (Amoo et al., 2009; Biswas et al., 2013). Antibacterial bioactive compounds are prevalently either polar or non-polar when extracted in organic medium. Organic crude extracts of the plants showed enhanced antimicrobial activity than of aqueous extracts (Jeyachandran et al., 1995). Therefore, methanolic and ethanolic extracts of Aloe considerably reduced the growth of the
targeted bacterial strains. Similar results were reported by (Al-inke, (2011) and Egamberdieva et al., (2017).

The accumulative evidences have been reported that there was reduction in growth of negative gram bacteria (S. aureus, E. coli and K. pneumonia) and gram positive bacteria (M. luteus, E. aerogenes and B. subtilis). Aloe extracts were found to be ineffective against E. coli 1 and 2 when applied as different conc. while, Staphylococcus aureus and Streptococcus pyogenes showed their susceptibility towards all the treatments (Elisha et al., 2017). The highest IZ 13.9mm ± 1.3 was measured at 75 mg/ml of ethanolic extract of A. vera after 48 hours of incubation against S. pyogenes. However, the minimum inhibition zone was recorded at 25 mg/ml of aqueous extract of Aloe after 24 hours incubation against E. coli 1 and E. coli 2 that might be due to less solubility of organic constituents in water. It has been recorded that methanolic and ethanolic extracts of A. vera were enriched with ingredients like flavonoids, steroids, saponins and glycosidic compounds (Kumar et al., 2017). Due to these ingredients in Aloe inhibitory activity against bacteria has been found (Palombo et al., 2001; Sriram et al., 2010; Mostafa et al., 2018).

**CONCLUSION**

Based on data, it is concluded that Aloe extract in Methanol solvent caused greatest inhibition of S. pyogenes in comparison with other bacterial stains.

**REFERENCES**


Evaluation of antifungal potential of Wood Biochar against *Fusarium oxysporum* Schlecht

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**ABSTRACT**

Antifungal potential of biochar produced from wood was evaluated against *Fusarium oxysporum* Schlecht. Methanol was used to prepare biochar extract of which various concentrations were experienced *in-vitro* against tested fungal strain. All the applied concentrations of methanol extract i.e. 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8% effectively inhibited *F. oxysporum* growth. However, 7% and 8% concentrations were proved to be most efficient in retarding the test fungus growth upto 15%.  

Partitioning of biochar methanolic extract was done using some organic solvents such as n-hexane, chloroform, ethyl acetate and n-butanol in increasing the polarity order. These secluded fractions were further tested to evaluate their *in vitro* antifungal activity along with a reference fungicide (Mancozeb). Data obtained after 7 days of incubation depicts that maximum antifungal activity was observed in fungicide with 89% inhibition in the radial diameter of the test fungus. n-hexane and chloroform also effectively retarded the growth of *F. oxysporum*, whereas n-butanol and ethyl acetate fractions gave minimum activity. So it can be concluded that wood biochar has the potential to control *F. oxysporum*.

**Keywords:** Bioassay, *in vitro*, Organic fractions, Methanolic extract, Phytochemical analysis.

**INTRODUCTION**

*Fusarium oxysporum* Schlecht belong to Ascomycetes, caused wilt disease in large number of economically important crop plants. It mostly penetrates in the infected crop residues, soil and seeds through its chlamydospores and mycelium. It also resides even in absence of host on stem and root tissue which are buried for more than 6 years (Singh et al., 2008). The appearance of banana shaped conidia is the main distinguishing character by which it can be recognized (Summerell et al., 2010). *F. oxysporum* colonize in the xylem tissue of its host which results in blockage and breakdown of tissue, followed by yellowing and wilting of leaves and ultimately leads to tissue death (Bennett et al., 2008).

Several methods like cultural, chemical, biological and use of resistance varieties are documented in the literature to control this disease (Mcgovern, 2015). Application of thick layer of mulch on the soil surface is helpful in reducing the growth of soil borne fungal pathogens. The use of opposed microorganisms is one of the most beneficial alternative measure in order to establish an ecofriendly atmosphere devoid of disease causing fungal strains (Lugtenberg & Kamilova, 2009). *F. oxysporum* can greatly be reduce by using synthetic fungicides which include fuberidazole, thiabendazole, carbendazim, prochloraz, benzimidazoles, propiconazole, benomyl and thiophante preferably (Nel et al., 2007).

Soil improvement using compost and biochar of plants have vast potential to limit the growth of disease causing pathogenic fungal specimens like *Fusarium* species. Because compost is a natural product of organic residues known to be derived by decomposition of aerobic microorganisms (Bonanomi et al., 2007). On the other hand, along with the enhancement of soil environment by increasing carbon levels, biochar is recommended as a low-cost preference (Yao et al., 2011; Elaigwu et al., 2014). Biochar is rich in carbon produced during a process named as “pyrolysis” in the oxygen deficient environment (Sohi et al., 2010; Elad et al., 2011). During this process, carbonization level of feedstock increased...
followed by high temperature, which indicated by reduced hydrogen and oxygen amount, whereas increased carbon content yield more biochar (Harvey et al., 2011; Uchimiya et al., 2011). A mixture of biochar and compost resulted in attributing more positive effects on plant growth (Schulz & Glaser, 2012) due to availability of more beneficial microorganisms in the rhizosphere followed by enhanced reproduction rate (Graber et al., 2010). Different biochar products have different physical and chemical properties depending on the type of feedstock available, for example, herbaceous or grass species has lesser amount of biochar as compared to woody biomass as it has higher concentration of lignin, cellulose and hemicelluloses (Lupoi & Smith, 2012). The application of biochar plays an important role in retarding disease rigorousness of foliar pathogens by activating plant defense mechanism (Elad et al., 2010). Biochar is well known to boost cation exchange, bulk density, water holding capacity, pH and nutrient retention in soil (Atkinson et al., 2010).

Due to these beneficial characteristics of biochar, the present study is designed to investigate antifungal activity of wood biochar against *Fusarium oxysporum*

**MATERIALS AND METHODS**

**Collection of test material**

Biochar produced from wood was selected to evaluate the antifungal potential against *F. oxysporum*. Biochar of wood was collected from Agro climatology lab, University of Agriculture, Faisalabad, Pakistan. Biochar was ground thoroughly to fine powder through electric blender.

**Procurement of test fungus species**

*F. oxysporum* was procured from Fungal Culture Bank, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. The culture was maintained on 2% MEA (Malt Extract Agar) medium and stored in refrigerator at 4°C for future use.

**Antifungal bioassay**

Medium comprising Malt Extract Agar (MEA) was primed using 250 mL of conical flask by the addition of 2 g of both agar as well as malt extract in 100 mL distilled water. For the extraction of biochar, 100 mL of methanol was taken in which 20g of grinded biochar was soaked at room temperature for one week and after that filtration was carried out by means of muslin cloth which has been autoclaved for sterilization purposes. In order to minimize the volume upto 2g the filtrate was subjected for evaporation at room temperature. Gummy extract was then diluted for the purpose of making 20% stock solution by the addition of sterilized distilled water. Various concentrations viz. 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8% from biochar methanolic extract were prepared. Control treatment was without any extract. In order to avoid contamination from bacterial growth chloromycetin capsule was added.

Five millimeter discs were cut with sterilized cork borer from the margins of seven days old culture of test fungus and mycelial discs were then transferred to each flask. Three replicates were made for each treatment. After inoculation the flasks were allowed to incubate for 7 days at room temperature for fungus to grow (Hanif et al., 2017). Percentage growth inhibition was calculated by using the given below formula for fungal colonies:

\[
\text{Growth inhibition} (\%) = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100
\]

**Phytochemical analysis**

Phytochemical analysis of biochar methanolic extract was carried out by using the protocol of (Parekh & Chanda, 2007) for identification of various secondary metabolites.

**Partitioning of test material**

Twenty grams of biochar was extracted with 100mL methanol at room temperature. This extraction yielded the gummy methanolic mass of 0.624g. The gummy mass was partitioned with n-butanol, ethyl acetate, chloroform and n-hexane in increasing polarity order (Wheed et al., 2016).

**In vitro antifungal activity of organic fractions**

The isolated fractions n-hexane, chloroform, ethyl acetate and n-butaol along with a reference fungicide were tested against *F. oxysporum*. From each organic fraction and fungicide three concentrations viz. 1%, 2% and 3% were made to evaluate their antifungal potential (Sherazi et al., 2016).

**Statistical Analysis**

Analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT) was used to analyze data statistically using the protocol of Steel & Torrie, 1980.
RESULTS AND DISCUSSION

In the present study the probable antifungal activity of wood biochar methanolic extract was studied against soil borne wilt causing detrimental plant pathogen *F. oxysporum*. According to the gained results all the applied concentrations of methanolic extract of biochar retarded the test fungal growth. However, 7% and 8% were found potentially good as compared to control against *F. oxysporum* with 15% inhibition. While other tested fractions were found less affective as compared to control treatment (Figure 1). As far as biochar antifungal potential is concerned little work from the literature is available. Although the present findings were in agreement with the results of Harel et al. (2012) that the use of biochar activates the plant defense mechanism which enables them to face challenges resulting from infections caused by harmful fungal specimens like *Colletotrichum acutatum*, *B. cinerea* and *Podosphaera aphanis*.

Table I: Phytochemical analysis of Biochar

<table>
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<th>Sr. No.</th>
<th>Phytochemicals constituents</th>
<th>Observations</th>
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<tr>
<td>1.</td>
<td>Tannins</td>
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</tr>
<tr>
<td>2.</td>
<td>Phlobatannins</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>Glycosides</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Terpenoids</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Flavonoids</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Presence  
- Absence

The phytochemical tests of this methanolic extract of wood biochar were performed (Table 1). The results indicated that all the phytochemicals including glycosides, alkaloids, tannins, saponins, terpenoids, phlobatannins and flavonoids were found absent in test material. Biochar is produced as a result of high heat pyrolysis, which can be a reason of absence of phytochemical. This might be the first report on phytochemical analysis of biochar as there was no previous literature available.

Partitioning of biochar methanolic extract resulted in the gummy masses of *n*-hexane (1.6 g), chloroform (1.1 g), ethyl acetate (0.4 g), *n*-butanol (1.2 g). *In vitro* bioassay with each organic fraction and fungicide was performed. Results showed that fungicides fraction was found most effective against *F. oxysporum* in comparison to other fractions (Figure 2). Highest growth reduction was given by fungicides and its all applied concentrations i.e., 1%, 2% and 3% reduced test fungal colony growth upto 86% and 80%, respectively. Other fractions also effectively inhibited the test fungal growth; among them 1%, 2% and 3%, concentration of chloroform showed 28%, 34% and 26% inhibition respectively. Tested concentrations of *n*-hexane fraction also showed 22-34% inhibition while *n*-butanol fraction concentrations was found least effective.

Elad *et al.* (2010) described that soil amended with biochar activated plant defense mechanism against soil microbes. This amendment effectively suppressed the infections caused by foliar pathogens, *Botrytis cinerea* and *Leveillula aurica* in tomato and pepper, respectively. Elmer & Pignatello (2011) reported that biochar also proved beneficial in minimizing root infections caused by *F. proliferatum* and *F. oxysporum*. On the basis of above findings the present study, can be concluded that wood biochar contain antifungal properties against *F. oxysporum*. 
**Fig. 1:** Effects of methanolic extract of Biochar on *in vitro* growth of *F. oxysporum* Schelecht. Vertical bars show standard errors different letters show non-significant difference.

**Fig. 2:** Effect of different concentrations of Biochar extract on *in vitro* growth of *F. oxysporum* Schelecht.
REFERENCES


Optimization of sewage sludge mixing with soil to attain promising neutraceuticals attributes in the *Lagenaria siceraria* L. (Kaddu) Plant

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ARTICLE INFORMATION

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<th>ABSTRACT</th>
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<td>Received in revised form: 04-12-2018</td>
<td>Disposal of sewage sludge is a critical issue worldwide for the environmentalists. There are many methods for its disposal. The most suitable and economical method is its land application. <em>Lagenaria siceraria</em> L. plant was grown in various amendments of soil and sewage sludge to check the tolerance of the plant and to select the optimized mixing of sewage sludge with soil for this particular plant. Estimation of biochemical parameters like chlorophyll content, carotenoid content, and protein content along with total biomass of the plant showed that the soil mixed with 20% sewage sludge showed maximum growth of the plant. Meanwhile, antioxidants like TPC, Ascorbic acid content and antioxidant potential (DPPH, ABTS, FRAP) were also measured and maximum values were found in 20% to 40% of sewage sludge. Hence, sewage sludge may be exploited for the useful purposes in a controlled manner.</td>
</tr>
<tr>
<td>Accepted: 04-01-2019</td>
<td><strong>Keywords:</strong> Sewage Sludge, <em>Lagenaria siceraria</em> L., Antioxidant potential</td>
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Original Research Article

INTRODUCTION

*Lagenaria siceraria* L. belongs to Cucurbitaceae family, its common name is bottle gourd (Urdu: Kaddu) and it is used as vegetable grown in Asia and Europe (Kubde *et al.*, 2010). There are 118 general and 825 species of this family (Clarke *et al.*, 2006). It contains many essential nutrients e.g, bottle gourd seeds are rich in protein (39.5 kg/100 kg of sample) and fat (46.1 kg/100 kg of sample). Moreover, minerals like potassium, magnesium and phosphorus are also present in this plant. Phenolic compounds such as, catechin, ascorbic acid and naringenin are also found in the fruit of this plant. (Ghule *et al.*, 2006). It was found that *Lagenaria siceraria* L. possessed antioxidant activity, central nervous system activity, anti-inflammatory activity, anti diarrheal activity, antimicrobial activity, cytotoxicity and anticancer activity (Palamthodi & Lele, 2014).

Rapid industrialization and massive increase in the population resulted in the generation of gigantic amounts of wastewater. Pretreatment of wastewater resulted in the generation of a solid residue called sewage sludge. Disposal of sewage sludge is an important issue for the environmental scientists. Utilization of sewage sludge for agricultural purposes is a very common practice worldwide. Sewage sludge has been reported to contain many useful contents which on mixing with soil enhance properties of the soil like bulk density, porosity, pH, mineral composition. On the other hand it may contain organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), detergents and remains of pharmaceuticals discharged by household activities and toxic metals released by industries which limit its application for the agricultural purposes. Therefore, controlled and optimized amounts of sewage sludge must be used for each plant grown in it as various species of plants have different tolerance level. Objective of this study is to enhance the growth, nutritional attributes and antioxidant potential of *Lagenaria siceraria* L. plant by optimizing the mixing of sewage sludge with soil.

MATERIALS AND METHODS

Preparation of soil amendments with sewage sludge (experimental setup)
Six different proportions of sewage sludge and soil (0% S.S, 20% S.S, 40% S.S, 60% S.S, 80% S.S and 100% S.S) were made (10 kg weight/pot) in triplicates respectively in the agricultural fields of University College of agriculture, Khushab Road, University of Sargodha, Sargodha. Seeds of *Lagenaria siceraria* L. (Kaddu) plant were sown in each pot and monitored for 75 days till harvesting.

**Physical growth parameters of plant**

Shoot length, number of leaves and size of leaves were measured for total biomass determination of fresh weight and dry weight as well. Biomass (FW, DW) of the plant *Lagenaria siceraria* L. grown in various compositions of soil and sludge were measured (g/plant) (Wang *et al.*, 2006).

**Chlorophyll and carotenoid contents**

For chlorophyll estimation about 1 g of mature leaves for each sample were taken and dipped in cold acetone (80%) for the period of 3 days in dark at 4ºC following method of Lichtenthaler, (1987). UV-Visible spectrometer was used for measuring absorbance of resulting extracts at 645 and 663 nm for the quantification of chlorophyll content and estimation of carotenoid was done at 470 nm (Wong, Li, & Wong, 1996).

**Protein contents**

500 mg of each plant sample was finely powdered and 5-10 mL of buffer (pH = 9) was added into each sample and centrifuged. Supernatant was used for measurements. Working standards were taken 0.2, 0.4, 0.6, 0.8 and 1 mL in five test tubes. Sample extract (0.1 mL) was also taken in each test tube. Make volume up to 1 mL for each sample and working standard with distilled water. A mixture (50 mL of 2% sodium carbonate in 0.1N Sodium hydroxide, 1 mL of 0.5% Copper sulphate in 1% potassium sodium tartrate and 5 mL of Alkaline Copper solution) was added in each test tube and allowed to stand for 10 min. after proper mixing. Folin-ciocalteau reagent (0.5 mL) was also added to each sample and incubated at room temperature for 30 min under dark. Protein content was estimated at 600 nm spectrophotometrically (Sadasivam & Manickam, 1992).

**Heavy metals content in plant**

Atomic absorption spectrophotometer (Shimadzu 7000 F) was used for the estimation of heavy metals in the fruit of *Lagenaria siceraria* L. for the toxicity evaluation of all the plants. (Sposito *et al.*, 1982) (Benbrahim *et al.*, 2006).

**Quantitative determination of ascorbic acid**

Indophenol titration method was used for the determination of ascorbic acid content in all parts of the plants (*Lagenaria siceraria* L.). Each methanolic extract (0.3 mL) was homogenized in metaphosphoric acid (8%) and 20 mL of glacial acetic acid (3%) solution. This mixture was titrated against 2, 6 dichloroindophenol till the pink color sustained. Results were presented as mg ascorbic acid/g of the powdered samples (Sims & Kline, 1991).

**Extraction and estimation of total phenolic contents**

For the determination of total phenolic content in all parts of plants (*Lagenaria siceraria* L.) extracts, FC reagent was used. For this purpose, 142 mL of Na₂CO₃ (7.5%) and freshly prepared FC reagent (800 μL) were mixed in diluted plant extract (200 μL) of each sample. All the mixtures were diluted with distilled water (7 mL). Solutions were kept for 2 h in dark under normal conditions for the completion of reaction. The absorbance of each sample was measured at 765 nm spectrophotometrically. Gallic acid was used as standard and results were presented as gallic acid equivalent. Results were obtained in triplicates and were averaged (Polshettiwar, Ganjiwale, Wadher, & Yeole, 2007).

**DPPH- scavenging assay**

A previously documented method was applied for the estimation of free radical scavenging activity of all the parts of plants (*Lagenaria siceraria* L.) for this purpose, each plant extract (2mL) was added to 5mL freshly prepared solution of DPPH (2, 2- diphenyl-1-picrylhydrazyl). Change in absorbance at 515 nm was recorded at different intervals of time like 0, 0.5, 1.0, 2.0, 5.0 and 10 min. (up to 50%). Remaining concentration of DPPH stable radicals was determined using standard curve. Capability of each plant extract was evaluated by noting absorbance at 515 nm after 5 min. Results were expressed in IC₅₀ (mg/mL) (S. Iqbal, 2005).

**ABTS⁺ scavenging assay**

Antioxidant activity of all parts of the plants was also measured using reported method called as ABTS⁺ radical cation scavenging assay. A filtrate was prepared by passing 2, 2’-azino-bis (3-
ethylbenzthiazoline-6 sulphonic acid) aqueous solution (5mM) through manganese oxide (oxidizing agent) on filter paper. Excess MnO$_2$ was removed from filtrate using fisher band membrane of 0.2 mm. Methanolic extracts of all the parts of plant were diluted with 5mM phosphate buffered saline (pH=7.4) to attain absorbance of 0.700 at 734 nm spectrophotometrically. Absorbance of all samples was measured after 10 min of mixing each extract (1mL) and ABTS$^+$ radical cation solution (5mL) at ambient temperature. Phosphate buffered saline (pH=7.4) was used as a control. Results were expressed as μmole Trolox equivalent/g DW for each sample. All readings were taken in triplicates (S. Iqbal et al., 2012).

**Ferric reducing antioxidant power (FRAP)**

Antioxidant activity was estimated using FRAP assay. Evaluation was done by taking 40 mM acetate buffer (pH 3.6), 20 mM ferric (III) chloride and TPTZ- tripyridyltriazine (10 mM in 40 mM HCl) were taken and combined in a ratio of 10:1:1 respectively. Crude methanolic extract (50 microliter) of all parts of *Lagenaria siceraria* L. were added to the above mentioned FRAP reagent (2mL) for each sample. Similar amount of FRAP reagent were added to the blank (Methanol). Absorbance of each sample was recorded spectrophotometrically at 593nm. All readings were taken in triplicates and results were expressed in mM Trolox equivalents in accordance to standard curve of Trolox (S. Iqbal et al., 2007).

**RESULTS AND DISCUSSION**

**Effect on the total biomass of *Lagenaria siceraria* L. grown in pure and amended Soil**

*Lagenaria siceraria* L. also showed prominent variation in total biomass with the change in sewage sludge proportion in soil. *Lagenaria siceraria* L. showed (Fig. 1) maximum yield in 20% amendment of sewage sludge with soil which is 677g fresh weight per plant and minimum fresh weight of the plant were estimated in the plant grown in pure sewage sludge. It reveals the sewage sludge as a potential nutrient resource along with its hazardous and toxic nature. (Gwenzi et al., 2016).

**Chlorophyll and carotenoid contents**

Chlorophyll a photosynthetic pigment has been reported to increase in the mustard plant when grown in the soil amended with mixed industrial effluents. (Singh & Rathore, 2018). Chlorophyll content has also been reported to increase in *Brassica nigra* L. when grown in amended soil (Karak et al., 2013). In our results, (Table I) maximum amount of the photosynthetic pigments estimated in the plants grown in 20% to 40% amendment of sewage sludge with soil ranging from 4.75 to 4.88 mg/g for chlorophyll a and 1.45 to 1.52 mg/g for carotenoid. Higher amendments showed negative results as chlorophyll and carotenoid content decreased predominantly.
Quantitative determination of ascorbic Acid

Ascorbic acid content was found (Fig. 3) maximum in the 40% sewage sludge amendment with the soil in the *Lagenaria siceraria* L. plant ranges from 12 to 14 mg/g in all parts of the plant. This variation featured the change in the soil characteristics and its fertility with the change in the amount of the sewage sludge in the soil and revealed the effect of soil amendment with sewage sludge.

Table I Chlorophyll content (Chl *a* and Chl *b*), Chl *a* / Chl *b* ratio and Carotenoid content in *Lagenaria siceraria* L. (Leaves)

<table>
<thead>
<tr>
<th>Sludge %</th>
<th>Chlorophyll <em>a</em> (mg/g FW)</th>
<th>Chlorophyll <em>b</em> (mg/g FW)</th>
<th>Chl <em>a</em> / Chl <em>b</em></th>
<th>Carotenoids (mg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>3.34±0.44</td>
<td>1.14±0.11</td>
<td>2.65±0.03</td>
<td>1.27±0.07</td>
</tr>
<tr>
<td>20%</td>
<td>3.46±0.24</td>
<td>0.88±0.14</td>
<td>2.44±0.07</td>
<td>1.38±0.11</td>
</tr>
<tr>
<td>40%</td>
<td>4.75±0.61</td>
<td>0.97±0.12</td>
<td>2.14±0.02</td>
<td>1.45±0.04</td>
</tr>
<tr>
<td>60%</td>
<td>4.88±0.01</td>
<td>1.23±0.11</td>
<td>2.26±0.12</td>
<td>1.52±0.08</td>
</tr>
<tr>
<td>80%</td>
<td>3.81±0.08</td>
<td>0.84±0.09</td>
<td>1.92±0.17</td>
<td>1.64±0.05</td>
</tr>
<tr>
<td>100%</td>
<td>3.73±0.02</td>
<td>0.78±0.05</td>
<td>2.12±0.19</td>
<td>1.55±0.02</td>
</tr>
</tbody>
</table>

Protein contents

Protein content in root shoot and leaves of the mustard plant have been reported to be increased at 50% amendment in 60 days of growth period (Ahmed et al., 2017). Protein content in roots, shoots, leaves and fruits of *Lagenaria siceraria* L. plant were found maximum in 60% amendment of sewage sludge with soil it was found 71, 88, 79 and 117 mg/g respectively in all parts (Table II). It drastically decreased at higher amendments of sewage sludge with soil such as at 80% and in pure sludge. This result proved the presence of toxic metal ions in the higher amendments of the sewage sludge (Wen et al., 2016).

Table II Protein content (mg/g) in various parts of the *Lagenaria siceraria* L.

<table>
<thead>
<tr>
<th>Sludge %</th>
<th>Roots</th>
<th>Shoots</th>
<th>Leaves</th>
<th>Fruits</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>55±0.66</td>
<td>57±0.55</td>
<td>64±1.12</td>
<td>97±0.54</td>
</tr>
<tr>
<td>20%</td>
<td>63±0.56</td>
<td>61±0.99</td>
<td>67±0.43</td>
<td>99±1.32</td>
</tr>
<tr>
<td>40%</td>
<td>71±0.32</td>
<td>88±0.45</td>
<td>79±0.66</td>
<td>117±0.57</td>
</tr>
<tr>
<td>60%</td>
<td>75±0.15</td>
<td>82±0.19</td>
<td>78±0.85</td>
<td>112±0.93</td>
</tr>
<tr>
<td>80%</td>
<td>69±0.66</td>
<td>71±0.12</td>
<td>67±0.43</td>
<td>94±0.45</td>
</tr>
<tr>
<td>100%</td>
<td>54±0.81</td>
<td>63±0.33</td>
<td>59±0.77</td>
<td>88±0.33</td>
</tr>
</tbody>
</table>

Toxicity evaluation of the plants

Heavy metals in the edible parts (Fruit) of the plant were quantified. All heavy metals estimated (Fig. 2) are in the permissible limit in the fruit of all the plants. So, there is no phytotoxicity found in the plant. Thus, edible part of the plants grown in the amended soil is recommended to be used for human being. However, repeated application of the sewage sludge in the soil may cause phytotoxicity. Therefore, optimized and controlled amount of the sewage sludge is required to apply for the best results with zero toxicity (Fu et al., 2018).
Ascorbic acid (Vitamin C) content (mg/g) in *Lagenaria siceraria* L. in all parts of the plant. The content was determined using Folin-ciocalteu based spectrophotometric assay. Total phenolic content in all parts of *Lagenaria siceraria* was optimum in 60% proportion of the sewage sludge in soil ranges from 240 to 250 mg of GAE/100g DW in all parts of plant (Fig. 4). Results revealed that increase in total phenolic content at certain levels in both the plants is due to the presence of organic matter, and higher amounts of macronutrients like N, P, K in the amended soil (Valifard et al., 2014). Conversely, elevated amount of heavy metals at higher rates of sewage sludge may inhibit the production of phenolic content by producing free radicals and other reactive oxygen species.  

**DPPH free radical scavenging activity**

*Lagenaria siceraria* L. showed (Fig. 5) maximum IC$_{50}$ value in the extracts (root, shoot, stem, leave) grown in the soil with 60% sewage sludge and the results found in the range of 18 to 20 IC50 mg/mL and this value decreases with the increase in the sewage sludge proportion in the soil. Thus, we can deduce that sewage sludge mixing showed its impact on the antioxidant activity with the change in the sewage sludge proportion in the soil (Krol et al., 2015).

**ABTS$^+$ Radical scavenging assay**

The scavenging activity of the methanolic extracts of the various parts of the *Lagenaria siceraria* L. have been shown in the Fig. 6. It is clear that ABTS$^+$ scavenging activity in all the parts of the plants increased up to the 60% S.S in the soil (7 to 9 μmole Trolox equivalent/g DW) while at higher percentage of sewage sludge with soil it is found to be decreased markedly.
CONCLUSION

Sewage sludge has been proved as a rich source of minerals and nutrients by altering the soil properties, which ultimately affects the nutraceuticals and antioxidant potential of the *Lagenaria siceraria* L. However, it is suggested that sewage sludge mixing with soil must be optimized as it may add phytotoxicity and other adverse effects to the plant. For *Lagenaria siceraria* L. 20% sewage sludge with soil is a best amendment.

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First report on Potato Spindle Tuber Viroid (PSTVd) from field grown infected Potato plants (Solanum tuberosum) in Pakistan

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ABSTRACT

The degradation of RNA is main hindrance for dealing with fine quality RNA for its downstream processing. Mostly RNAs are degraded as tissues are taken from fields, therefore the protocol for PSTVd RNA isolation was optimized and improved for obtaining pure and high quality RNA having an increased and nondegradable life from infected plants of Potatoes. The present study was aimed to confirm the presence and identification of potato spindle tuber viroid (PSTVd) on potato plants in Pakistan. RNA was extracted and purified using optimized Trizol reagent and RNeasy Miniplant Kit. The RNA obtained was converted into cDNA on the same day and amplified. The identification of PSTVd was carried out by Sequencing and BLAST. The advanced optimized Rapid Trizol reagent method was comparatively found best and reproducible as compared to the second method. The Sequencing and BLAST confirmed the presence of Pakistani PSTVd (Accession # MK.303578) in infected potato plants.

INTRODUCTION

Potato spindle tuber viroids was first identified small circular single stranded RNA viroid causing infection in many crops, weeds, ornamental plants, etc. This is a representative member of Pospiviroidae family (Bostan et al., 2004). Its genome is consisting of 359 nucleotides forming a rod-like conformation, having five distinct domains. All domains have been reported with different functions (Candresse et al., 2010). It was general perception that only pathogenic Domains (P) are involved for inducing infection in plants (Atsushi et al., 2013). Viruses and Viroids are responsible for 40-50 billion of field losses every year effecting stored and cultivated crops equally, proving themselves one of the major hurdle in effective food production and supply (Owens, 2009). The short-lived vegetables and herbaceous annual crops, e.g. Tomato, Potato and Capsicum etc., grown by true seeds, display maximum losses every year effecting stored and cultivated crops equally, proving themselves one of the major hurdle in effective food production and supply (EFSA, 2011). There is a tremendous increase in number of plant viroids identified in last three decades, thus creating attention of global community to protect their fields and farms from exotic and imported pathogens (Anna, 2013). Two decades earlier, it was a general opinion that any organism without a protein coat could not replicate itself, even with the help of host cell protein. However, discovery of PSTVd proved it wrong. The PSTVd was the first viroid identified, as physical entity, native to Australia and posing serious threat to world potato economy. Approximately 32 plant families have displayed sensitivity to viroids (Kovalskaya and Hammond, 2014).

The complicated plant pathogen and host interaction process is the key behind the development of disease signs and symptoms. The degree and type of infection depend on systemic transfer efficiency. PSTVd exhibits mild to severe symptoms in plants. Diagnoses of PSTVd depend on symptoms, appearing on leaves or stems of infected plants like Chlorosis, stunted growth in potatoes and purpling of leaves (Flores, 2009). By the time potato has become an important cash crop for both Pakistani farmers and consumers. It is the fourth most significant crop by volume and yield of production and gives highest returns to field growers (Barbara et al., 2012). Almost 86% of potato area and production in Pakistan is achieved from Punjab. Pakistan is self-sufficient in potatoes for country consumption. This is a tasty, nutritive and highly digestible vegetable with 75 % water contents. The average yield of potato is 30-50 tons/ha which it depends upon the location, variety and cultural practices (FAO, 2016). Although a lot of work has been performed on isolation of RNA from infected plants tissues, but this is first report of Isolation of PSTVd RNA from infected plants in Pakistan. The extraction of RNA is always a big
problem and challenging in university laboratories. Moreover, it is not always possible to follow published protocols because of expensive and complicated apparatus discussed in research papers. The fast efficient protocol for isolation of PSTVd was optimized in the present work and found very short and effective protocol completing in less than three to four hours as compared to RNAeasy Miniplant Kit. Another plus point of the discussed method is that extracted RNA can be stored for longer periods without converting into cDNA. For completion of experiment required RNA was converted into cDNA.

**MATERIALS AND METHODS**

**Selection and collection of infected plants**

Plants were collected on the basis of symptoms discussed in the work of Owen, 2009. The fresh leaves were analyzed with yellowing and spindle like formation in Potato. The leaves were plucked, covered in Aluminum foil and immediately shifted to liquid Nitrogen small container to save RNA from degradation. Infected plants were collected from different geographical areas in Pakistan. Some basic signs & symptoms were also studied as morphological markers to identify PSTVd in infected plants.

**RNA extraction with RNeasy plant mini kit**

Total RNA was extracted with the modified protocol of Kolonko et al., 2006, using RNeasy Plant Mini Kit. The frozen leaves were ground and poured into falcon tube, added 400µl of ribozol reagent was added into it and mixed properly. These tubes were incubated at room temperature at 34°C for 30 minutes. The tubes were put on ice and after fifteen minutes, the chilled tubes were centrifuged at 15000rpm at 15°C for 5 minutes. Two clear layers were formed and the supernatant layer was transferred to fresh tubes, each containing 100µl of chloroform. The tubes were vortexed for a minute in fume hood and incubated at room temperature for 60 min. The incubated samples were centrifuged again for 15 min at 10000rpm. The clear aqueous phase was transferred to new tubes, each containing and 200µl of isopropanol. The mixing of samples was done by inverting tubes many times and then incubating again for third time. These tubes were centrifuged for 10 min at 12000 rpm. The supernatant was removed and 200µl of 75% Ethyl alcohol was added and RNAase free water was used in order to wash pellet. The final samples in tubes were spun at 7500 rpm for 10 min at 4°C. The clear RNA Pellet was saved and dried for 5 min. Concentration of RNA was measured by spectrophotometry at 260nm Nanodrop 2000/2000c. The extracted RNA was preserved at -80°C.

**RNA extraction with modified Trizol protocol**

The infected frozen plant tissues were ground in 500 µl of Trizol reagent and then 100 µl of chloroform reagent was added. The total sample was vortexed for 20 seconds and incubated at room temperature for almost 30 minutes. Then it was centrifuged at 13000rpm for 5-10 minutes. Two phases were formed, lower red interphase and colourless aqueous phase. The upper phase was collected and 250µl of cold Isopropanol was added at room temperature. This was again centrifuged at 13000rpm 4°C for 20 min. The pellet was collected and 500 µl of 75% ethanol was added and centrifuge for three min at 750rpm. The remaining liquid was discarded and pellet was dried and suspended in 500 µl of Trizol reagent.

**cDNA Synthesis**

For completion of experiment Extracted RNA was converted in to cDNA on the same day using GScript First strand synthesis Kit (Cat No MB 305-0050) according to manufacturer's Instructions. Extracted RNA solution was added into eppendorf with following kit’s components i.e. Oligo (dT) 1μl, dNTPs 1μl, 13μl of Nuclease free water and mixed properly. Then it was centrifuged at 13500 rpm for 5 min and shifted to pre warmed water bath for almost 3-5 min at 65°C. As incubation was completed, the whole mixture was spun shortly and shifted on ice. The available 5x is strand buffer (4µl) was added, soon after buffer's addition the Gscript Rtase and DTT (0.1M) were added as 1µl Respectively (Owens & Baumstark, 2007).

**Agarose gel run**

The quality of cDNA was checked on 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. The quantity of cDNAs was also noted with spectrophotometry. All samples were found between 1-2 nm. The Quantification of cDNA was performed with Thermo Fisher Scientific V1.0. The large peaks at260/280nm were obtained for purified sample. The Visualization of bands were done in gel documentation system and was photographed and fine quality bands were seen and scored (Fig A) (Gozmanova et al., 2003)
**Sequencing**

The presence of PSTVd was confirmed by BLAST and sequencing. The obtained FASTA sequence showed 100% similarity with other reported accessions # (U.23058.1 and A.F459007.1) in NCBI BLAST results (Fig B-1). The amplified sequences were inserted into a vector according to protocol of Vachev et al., 2010 and sent for sequencing to Bio Basic Company.

**Amplification of PSTVd**

The PSTVd cDNA was further amplified with the primers designed by Vachev et al., 2014) and sent for sequencing. The amplified sequence also confirmed the presence of this deadly pathogen in the infected samples.

**RESULTS**

The two methods of RNA isolation, RNA Extraction with RNeasy Plant Mini Kit and RNA Extraction with Modified Trizol Protocol from PSTVd were analyzed comparatively. It was noted that the second modified methods was found best and easy to use then the former. (Fig.1). This modified Trizol method was also found efficient in time saving and quality of RNA. The RNA was stored in 2μl of Trizol reagent for future use. This property of optimized Trizol reagent also found efficient over RNeasy Plant Mini Kit method. The prior addition of β-Mercaptoethanol (1-2 μl per ml of Trizol reagent) also gave best quality RNA. The amplified sequence also gave positive, compact and distinct bands for PSTVd (B).

**Fig. 1:** RNA from infected samples: 1st well: 100 bp ladder, 2nd 3rd well: RNA with RNeasy Plant Mini Kit; 4th well Modified Trizol reagent. 5th and 6th well; stored RNA (Trizol reagent)

**Fig. 2:** Sharp bands of amplified PSTVd sequence with Modified Trizol methods

**Fig. 2a:** NCBI blast results of PSTVd FASTA showing 100% and 99% similarity with other reported sequence.
**DISCUSSION**

The extraction of PSTVd has been reported earlier by many researchers in different countries (Constable et al., 1996; Boonham et al., 2004; Singh et al., 2006; Di Serio, F. 2007; Qiu et al., 2016) but the present PSTVd isolation is first report of presence of PSTVd in Pakistan. The already reported methods of PSTVd RNA isolation were also found best but those all need highly equipped Laboratories, which is not possible in University Laboratories of progressing and low income countries in Pakistan. This presented work is reported as the modification of different researchers’ work (Ding, Itaya, 2007; Guner et al., 2012). The present research is easy, simple and economic time saving isolation of PSTVd RNA from infected plants. The modification of extraction buffer by adding b-Mercaptoethanol in trizol reagent improved the isolation as compared to RNeasy Plant Mini Kit protocol (Mackie, 2015). It was worthy to note that isolated PSTVd RNA when amplified and sequenced showed complete match with NCBI BLAST sequences (Vachev et al., 2014). The obtained sequence was deposited in Gene bank (Accession # MK.303578) and Blastn searches at NCBI website demonstrated that this PSTVd isolate shared 100% similarity with Accession # U.23058.1 in *Solanum tuberosum*. These results have confirmed the presence of PSTVd in infected potatoes plants in Pakistan. This research could be served as a basic solid reference for planning and designing more explanatory research in viroids functions and structures for this pathogen control in Pakistan (PARC, 2010). The attack of pathogens always effected and slowed down the economy of agricultural countries like Pakistan. It is need of time to diagnose and identify actual cause of plant diseases (Hammond, 2001). The infection symptoms of virus and viroids often confused with each other therefore proper extraction and confirmation by sequencing is vital step for identification of viroids (Vachev et al., 2014).

In present work the infection symptoms were clearly observed and distinguished as morphological markers to identify PSTVd in infected plants according to literature. Three main signs and symptoms of PSTVd disease i.e yellowing, purpling of leaves with stunted stem growth were focused during present study (Mascia, 2009). It was also noted during infection plants collection that high grade temperature and light tropical regions of Pakistan also favoured presence of PSTVd. The previous reports of PSTVd instability were also taken into accounts (Varadarajan, 2003; Mumford, 2004; Ward et al., 2010; Vachev et al., 2014; Mertelik et al., 2010 and shelf and storing condition of PSTVd RNA and cDNA were also improved during present study. The knowledge and findings about PSTVd has been discussed a lot during last decades in European countries.

However more need to be done, Like how this pathogen overtakes host plant’s machinery to overtake advantage. It is also necessary determine source and route of PSTVd entry into crops. The present work would open up new avenues and eras for presence of PSTVd (Pathogen) and disease management.
CONCLUSION

The presented study has proved as an outstanding and efficient method for extraction of PSTVd RNA, its cDNA synthesis, amplification and storage. The Isolation of PSTVd from infected plants is a new addition to existing research.

Conflict of interest’s statement

Authors declare that there is no conflict of interest for publishing this study.

ACKNOWLEDGEMENT

The Molecular genetics and Plant Biotechnology Lab in the Department of Botany, Lahore College for Women University provided all the necessary items during present studies. Some part of research was also executed under the Guidance of Sir Dr. M. Ashfaq and help of hard working student Mubarak Ali Anjum in Institute of agriculture, University of Punjab. We are very thankful to kind cooperation of different students of both labs.

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Optimization of cultural conditions for enhanced production of laccase by *Aspergillus flavus* Maf 0139

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**ABSTRACT**

Laccase enzyme has a range of biotechnological and industrial applications but its high cost hindered its use. Development of low-cost biotechnological process for laccase production has gained great attention. Present study is concerned with the enhanced production of laccase by *Aspergillus flavus* Maf 0139. Five different cultural media were tested and best one was selected for laccase production. Different cultural conditions such as inoculum size, pH, temperature, incubation period, moisture content and inducer were studied. Medium components were further optimized by Plackett-Burman and central composite designs. Results showed that maximum enzyme activity (297±0.1 U/ml/min) was obtained by using AFM02 medium containing g/L: sawdust, 5.0; glucose, 5.0; peptone, 6.0; MgSO₄.7H₂O, 0.25; KCl, 0.5; KH₂PO₄ 0.025. Optimized conditions for maximum enzyme yield were inoculum size 2.5%, Temperature 25˚C, pH 4.5, incubation period 7 days and moisture content 60%. Eleven different inducers were evaluated but FeSO₄.7H₂O showed maximum enzyme production (2156±0.57 U/mL/min). By using these statistical techniques more than 13 folds increase in enzyme yield (4022±0.25 U/mL/min) was observed. It is concluded that cultural conditions have great influence on enzyme production and enzyme yield could be enhanced by using advanced statistical techniques.

**Keywords:** Multicopper oxidase, lignocellulosic waste, Solid state fermentation, extracellular enzyme

**INTRODUCTION**

Laccases are blue multicopper oxidases having potential to oxidize various phenolic and diphenolic substances and reduce molecular oxygen to water (Arora & Sharma, 2010; Songulashvili et al., 2016; Nguyen et al., 2016). Release of water as a byproduct increases its significance for its use in various industrial applications as a ‘green’ catalyst (Surwase et al., 2016). Use of inexpensive raw materials in the form of agroindustrial or lignocellulosic waste (Arora & Sharma, 2010; Kumar et al., 2013; Daassi et al., 2016) for enzyme production makes the whole process cost effective. Solid state fermentation is a better option for fungal cultivation as metabolite produced are more concentrated, economical purification procedures, easier product recovery and simple process (Pandey et al., 2000). One of the major advantages of solid state fermentation is high yield of enzyme (Ergun and Urek, 2017). Optimization of cultural conditions like inoculum size, pH, temperature, moisture content, incubation period play critical role in enzyme production. In contrast to an efficient but intricate and costly tool of bioengineering, addition of inducer either in the form of aromatic compounds and metal ions is supposed to be a simple and cost-effective approach to enhance enzyme yield (Levin et al., 2010; Baldarian & Gabriel, 2002; Hou et al., 2004). The optimization of medium components by one variable at a time is laborious and time consuming approach, often the effect of interaction between various components are overlooked. To overcome this problem advanced statistical experiments like Plackett Burman and Central Composite designs are effective to get information in minimum experimental runs (Yahya et al., 2016). Plackett-Burman design is used to evaluate the effective components of medium and significant factors obtained after screening were further optimized for their concentration by central composite design. Although extensive data has been reported on the enhanced production of laccase by white rot fungi but only limited work is available on brown rot fungi. Present study is designed to optimize the physicochemical conditions.
parameters to enhance the production of laccase enzyme from brown rot fungi.

**MATERIALS AND METHODS**

**Organism**

The strain *Asperillus flavus* Maf 0139 was previously isolated from decaying wood of *Mangifera indica*. Culture was maintained on freshly prepared potato dextrose agar (PDA) slants and stored in refrigerator at 4°C.

**Fermentation technique**

The extracellular laccase production from *Asperillus flavus* Maf 0139 was carried out in shake flask using five different fermentation media. Chemical composition of media components expressed in g/L.

**AFM01**: Sucrose, 10; peptone, 5.0; NH₄NO₃, 1.0; CaCl₂, 1.0; KH₂PO₄, 1.0; MgSO₄, 1.0; FeCl₃, 1.0; CoCl₂, 1.0; ZnCl₂, 1.0; KCl, 1.0; CuSO₄.7H₂O, 1.0; BaCl₂, 1.0; HgCl₂, 1.0; ZnSO₄, 1.0

**AFM02**: Sawdust, 5.0; Glucose, 5.0; Peptone, 6.0; MgSO₄.7H₂O, 0.25; KCl, 0.5; KH₂PO₄, 0.025

**AFM03**: Glucose, 10.0; KH₂PO₄, 0.025; Peptone, 2.0; Potato dextrose broth, 24; MgSO₄.7H₂O, 0.5

**AFM04**: Glucose, 10.0; L. Histidine, 0.5; NaCl, 1.8; NaNO₃, 1.8; KCl, 0.5; CuSO₄.7H₂O, 7.0; CaCl₂, 0.5; FeSO₄.7H₂O, 0.05; Glycerol, 7.5; KH₂PO₄, 1.0; MgSO₄.7H₂O, 0.5

**AFM05**: Rice bran, 5.0; Distilled water, 6.0Ml

These fermentation media were prepared in 250mL Erlenmeyer flasks. The flasks were autoclaved (HVA 110, JAPAN) for 15 min at 121°C. The medium was allowed to cool at room temperature and then inoculated with 2.5mL of fungal spore suspension under aseptic conditions and kept for seven days in an incubator at 25±1°C. After seven days of incubation the cultural broths of these five media were centrifuged (Hettic EBA 8S, GERMANY) at 6000 rpm for 15 minutes and the supernatant obtained was used for enzyme assay.

Different nutritional conditions and process parameters such as inoculum size (0.5-3%v/v) with interval of 0.5 units, temperature (20, 25, 30 and 35°C), incubation period (5, 7, 9, 11, 13 and 15 days), pH (3.5, 4.5, 5.5, 6.5 and 7.5), initial moisture content (10, 20, 40, 50, 60 and 70%) of the medium was established by adding salt solution before autoclaving the medium, effect of different inducers (Ethanol, methanol, isopropanol, acetone, Tween 80, CaCl₂.2H₂O, NaNO₃, MgSO₄.7H₂O, FeSO₄.7H₂O, glucose, CuSO₄.7H₂O, glycerine and Triton X) and their concentrations at 1, 1.5 and 2% w/v were optimized to scale up the laccase production.

**Laccase enzyme assay**

Laccase enzyme assay was performed according to the method of Khammuang & Sarnthima, (2007). The reaction mixture contained 0.5 ml of enzyme, 940 µl of 0.1 M sodium acetate buffer of pH 4.5 and 10µl of 10mM ABTS [(2, 2-azinobis (3-ethylbenzthiazoline-6-sulphonate) diammonium salt]. The reaction mixture was incubated at 30°C in water bath for 10 minutes. The rate of ABTS oxidation was measured spectrophotometrically at 420nm.

**Plackett-Burman Design**

For screening of optimized medium (AFM02) components Plackett- Burman design was applied. This experiment was conducted in 12 trials. Optimized medium has six components and the effect of each component on laccase production was studied by missing (-) one or two components in each trial as given in Table I. Each trial was performed in 250 ml conical flask. Medium was inoculated with fungal spore suspension and kept at 25°C for 168 hrs. in static conditions. Minitab version 18.0 was used for analyzing the data. All experiments were performed in triplicates and the mean of laccase production were used as response of experiment (dependent variable). Plackett Burman design is based on first order model as shown in equation 1.

\[
Y= \beta_0 + \sum \beta_i x_i \quad \text{(1)}
\]

Where,

- \(Y\) = Dependent variable (enzyme activity)
- \(\beta_0\) = Model intercept
- \(i\) = Variable number
- \(\beta_i\) = Variable estimated coefficient
- \(x_i\) = Independent variables

For determination of statistical significance of the model and significance of each term in equation, Student’s t test was used to determine the significance of regression co-efficient. \(R^2\) value evaluated the adequacy of the model. Variables having P value less than 0.1 had significant effect on enzyme production (Mathur et al., 2013; Yahya et al., 2016).
Central composite design

Central Composite design was applied to optimize the concentration of medium components selected in the Placket Burman design. The variables used in this experiment are shown in Table III. Effect of increase or decrease in concentration of significant factors of medium components was determined by enzyme activity (dependent variable). Validity of the model was evaluated by \( R^2 \) value which is Coefficient of determination (Sharma et al., 2017).

Statistical analysis

All results are the mean of triplicates and expressed as mean ± S.D using Microsoft excel 2010 and Minitab version 18.0.

Results and Discussion

Selection of Fermentation medium

Enzyme production varies according to the macro and micronutrients of the fermentation medium. To optimize the best medium for laccase production by Aspergillus flavus Maf 0139 five different fermentation media AFM01 (Vivekanandan et al., 2014), AFM02 (Daassi et al., 2014), AFM03 (Nadeem et al., 2014), AFM04 and AFM05 (Devi et al., 2012) were evaluated (Figure 1). It was observed that the basal medium (AFM02) having sawdust as a solid substrate gave maximum enzyme production (297±0.1 U/mL/min) whereas, the enzyme productivity in the other basal media i.e., AFM01, AFM03, AFM04 and AFM05 was 75±0.1, 19±0.15, 178±0.5, 155±0.2 U/mL/min, respectively. Our results are in close agreement with Daassi et al. (2016). Ado et al., 2018 also found sawdust as a best solid support for laccase production by Trametes sp.

Size of inoculum

Effect of inoculum size on laccase production was studied ranging from 0.5 to 3.0 % (v/v). Results showed that maximum laccase productivity (670±0.15 U/mL/min) was achieved with 2.5% size of inoculum as shown in Figure 2. Further increase to 3.0% resulted in decrease of the enzyme yield (480±0.2). Vantamuri & Kaliwal, 2016 reported that highest laccase enzyme production (1.4 U/ml) was obtained when 2000µl of inoculum was added in 5g of rice bran. Enzyme yield decreased with the increase in inoculum size due to the accumulation of toxic metabolites and rapid utilization of substrate. These results are in accordance with Deb et al. (2013).

Effect of temperature

The production of laccase from Aspergillus flavus Maf 0139 at 20-35°C temperature range revealed that maximum enzyme production (890±0.2 U/ml/min) was achieved at 25°C as shown in Figure 3. Increase in temperature to 30°C resulted decrease in enzyme production (760±0.1U/mL/min) and significant decrease in enzyme production (192±0.15U/Lmin) was observed with increase in temperature to 35°C. The increase in temperature decreases enzyme production because high temperature alters the composition of cell membrane and stimulates protein catabolism (Nadeem et al., 2014; Edae and Alemu, 2017). The trend of high laccase production at low temperature and decrease in production at high temperature was also reported by other workers (Adejoye and Fasidi, 2010; Patel et al., 2009; Ado et al., 2018).

![Fig. 1: Effect of cultural media on the production of laccase from Aspergillus flavus Maf 0139](image1)

![Fig. 2: Effect of inoculum size on the production of laccase from Aspergillus flavus Maf 0139](image2)

![Fig. 3: Effect of temperature on laccase production from Aspergillus flavus Maf 0139](image3)
Effect of pH

pH is an imperative factor and had great influence on enzyme production (Sivakumar et al., 2010). The fungus was cultivated at different pH ranging from 3.5-7.5. It was observed that laccase gave maximum enzyme production (896±0.1 U/mL/min) at pH 4.5 as shown in Figure 4. These results are in line with the Stoilova et al., (2010) who revealed that Trametes versicolor express maximum activity at pH 4.5. In another study, Trametes sp. gave maximum laccase production (2356 U/ml) at pH 5.0 (Ado et al., 2018). Further increase in pH decreases laccase production due to the fact that increase in pH is unfavourable for fungal growth. Increase or decrease in pH effected enzyme production due to changes in the structure of enzyme. Amount of soluble proteins and the fungal biomass gradually decreased with the increase in fermentation pH. These results are in accordance with other workers (Minussi et al., 2001; Yang et al., 2011).

Effect of inducer

The addition of inducer is an effective approach to increase enzyme production. Different inducers such as ethanol, methanol, isopropanol, acetone, Tween 80, Triton X, CaCl₂, MgSO₄, FeSO₄.7H₂O, CuSO₄.7H₂O and Glycerine were tested for their potential to induce laccase production (Figure 5). It was observed that in comparison to all other inducers, FeSO₄.7H₂O gave maximum enzyme production of 1156±0.15 U/mL/min. whereas addition of acetone and copper sulphate as inducer showed medium productivity of 761±0.1and 684±0.3 respectively. According to Fonesca et al. (2010) laccase production was induced by iron and copper ions through post translational and translational regulation. It has been reported that expression of fungal laccases is regulated by Fe²⁺ at transcription level (Akpinar & Urek, 2017). In high affinity iron (Fe) uptake the reductive iron assimilation system plays important role in Aspergillus. This assimilation system consists of Ferric reductase, ferrooxidas and Fe permease (Hass, 2012; Blatzer et al., 2011; Bailao et al., 2015). Further increase in the production of enzyme was studied by the addition of inducer at different concentrations (1%, 1.5% and 2%) as shown in Figure 6. The optimum level of inducer concentration was found to be 1.5% (w/v) and the amount of enzyme produced was (2156± 0.2 U/ml/min). Further increase in the concentration of inducer to 2% reduced the laccase production (184± 0.5 U/ml/min).

![Fig. 3: Effect of temperature on the production of laccase from Aspergillus flavus Maf 0139](image)

![Fig. 4: Effect of pH on the production of laccase from Aspergillus flavus Maf 0139](image)

![Fig. 5: Effect of different inducers on production of laccase from Aspergillus flavus Maf 0139](image)
Effect of moisture content

In solid state fermentation initial moisture content played a vital role and affected significantly on laccase production and substrate utilization. Laccase produced by Aspergillus flavus Maf 0139 was positively affected by high moisture content. Increase in moisture content from 30-60% remarkably increased laccase production. Maximum laccase production (2362±0.15 U/mL/min) was achieved at 60% moisture content as shown in Figure 7. However, increase in moisture content above 60% decreased laccase production (2318±0.3 U/mL/min) because porosity of the substrate was reduced due to increase in moisture content and also limiting oxygen transfer. In an earlier study it was reported that optimum moisture content was found to be 60% when the Pleurotus ostreatus was grown on wheat straw (Patel et al., 2009). Our results are consistent with Vantamuri & Kaliwal, 2016 who reported that 65% initial moisture content of the substrate (rice bran) was optimized for production of laccase by Marasmius sp.

Optimization of fermentation medium by Plackett- Burman and central composite design

Medium components for laccase production by Aspergillus flavus Maf 0139 in solid state fermentation were optimized by Plackett-Burman and central composite design. Statistical approach helped us to enhance enzyme yield resulting in the reduction of production cost making the fermentation process cost effective (Kaur & Satyanarayana, 2005). The screening of multiple independent variables in a single experiment is the main advantage of Plackett-Burman design. Independent variables (medium components) including sawdust, peptone, glucose, KH₂PO₄, KCl and MgSO₄.7H₂O were evaluated in six factors Plackett-Burman design (Table I) and result was that all these ingredients constituted positive effect

Time course fermentation

To determine the effect of incubation period on laccase production by Aspergillus flavus Maf 0139 shake flasks were incubated for different time intervals (5, 7, 9, 13, 15 days) at 25°C. On 5th day of incubation, the amount of laccase was 2658±0.25 U/mL/min and increased (3217±0.12 U/mL/min) after 7 days of incubation (Figure 8). Further increase in the time course did not enhance enzyme production and resulted in significant decrease (1040±0.15 U/mL/min) after 15 days of incubation. In an earlier study, maximum laccase production in Lentinus edodes and Ganoderma sp. was also obtained on the 7th day of incubation (Sivakumar et al., 2010). According to Nadeem et al., 2014 sharp increase in laccase production by P. ostreatus was observed from 2nd to 6th day of incubation.
on the production of laccase having maximum activity 2675±0.1 U/ml/min. The effect of each variable at significant level was determined by student t test (Table II).

Further, concentration of medium components was optimized by central composite design as shown in Table III. The components of trial, 11 showed positive effects on the production of enzyme and enzyme yield was 4022±0.25 U/ml/min. Central composite design for optimization of medium concentration has also been reported by other workers for different fungal strains (Gao et al., 2013; Vivekanadan et al., 2014; Bagewadi et al., 2017). Analysis of variance was performed in order to validate the regression model (Table IV).

Table I: Plackett-Burman experiment of sawdust supplements assigned different levels of factors. Fermentation conditions: inoculums size: 2.5%, incubation time: 7 days, temperature: 25˚C and medium AFM02

<table>
<thead>
<tr>
<th>Trials</th>
<th>Sawdust (g/L)</th>
<th>Glucose (g/L)</th>
<th>KH$_2$PO$_4$ (g/L)</th>
<th>KCl (g/L)</th>
<th>MgSO$_4$.7H$_2$O (g/L)</th>
<th>Peptone (g/L)</th>
<th>Enzyme Activity (U/mL/min)</th>
</tr>
</thead>
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<td>0.5</td>
<td>0.25</td>
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<td>0.5</td>
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<td>0.5</td>
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<tr>
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<tr>
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<td>0.025</td>
<td>-</td>
<td>0.25</td>
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Table II: Regression analysis for the Plackett-Burman design.

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<th>T-Value</th>
<th>P-Value</th>
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Coefficient of determination, $r^2 = 0.97$
Table III: Central composite design for five sawdust supplements, assigned different levels of factors. Fermentation conditions: inoculum size: 2.5%, incubation time: 7 days, temperature: 25°C and medium AFM02

<table>
<thead>
<tr>
<th>Trials</th>
<th>Sawdust (g/L)</th>
<th>Glucose (g/L)</th>
<th>KH$_2$PO$_4$ (g/L)</th>
<th>KCl (g/L)</th>
<th>MgSO$_4$.7H$_2$O (g/L)</th>
<th>Peptone (g/L)</th>
<th>Enzyme activity (U/mL/min)</th>
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Table IV: Regression analysis for central composite design

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</table>

Coefficient of determination, $r^2 = 0.84$
CONCLUSION

It is concluded that the isolated fungus Aspergillus flavus Maf 0139 is an active laccase producer under solid state fermentation rather than submerged fermentation. The present study proved that sawdust, a cheap and inexpensive lignocellulosic waste, can be utilized for the production of valuable enzyme. Optimization of cultural conditions and use of an appropriate inducer enhanced laccase production and its hyperproduction could be achieved by optimization of medium constituents in minimum experimental runs using advanced statistical techniques.

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Sugar industry effluents as a source of soil fertility and potential toxicological risk of heavy metals in food crop

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ABSTRACT
Concentrations of heavy metals such as manganese, zinc, lead and nickel in the environment are currently increasing, mainly due to human activities. Zinc is essential element for several biochemical processes in plants. Any of these metals, at high concentrations in soil, can cause severe damage to physiological and biochemical activities of plants. Scarcity of fresh water in agricultural area enforced farmers to use industrial effluent and domestic wastewater for irrigation purpose. Ramzan sugar mill industry located at Chiniot discharges high amount of effluent which is used by farmers for irrigation purpose. Current experiment was conducted in Sargodha, Punjab, Pakistan to assess the level of different heavy metals such as Mn, Ni, Pb and Zn in wheat variety (Chagi-4) irrigated with varying quantity of sugar industry effluent. The water, soil and wheat grain samples were analyzed for heavy metals by Atomic Absorption Spectrophotometer. Concentrations of Mn (0.29-0.61), Ni (0.79-1.02), and Pb (0.01-0.42) mg/L in water samples were higher than the permissible limit of Mn (0.2), Ni (0.20), and Pb (0.1) mg/L given by FAO, while value (mg/L) of Zn (0.61-0.80 mg/L) was within the acceptable limit recommended for irrigation. In grain samples, values of all heavy metals such as Mn (0.18-0.75), Ni (0.32-0.77), and Zn (0.52-0.98) mg/kg were within acceptable range of Mn (500), Ni (67), Zn (99.4 mg/kg) suggested by FAO/WHO except for Pb whose concentration (0.19-0.83) mg/kg exceeded the permissible limit (0.3 mg/kg) given by FAO/WHO. The values of all heavy metals in water were beyond the acceptable limit but in wheat grains concentrations of heavy metals were within acceptable limit except for Pb, which indicate the lesser transfer of these metals from soil to the wheat plant. The analysis reveals that regular monitoring of sugar industry effluent is necessary to prevent the excessive buildup of metals in food chain which has broader implications in sustainable agricultural water management.

Keywords: Industrial wastewater, Bioconcentration factor, Triticum aestivum

INTRODUCTION

Study of heavy metal pollution is gaining much more importance all over the world. Some heavy metals essential for growth of plants and animals are called micronutrients. Other heavy metals are non-essential for growth of plants and animals and cause various problems in plants and animals when present in excess amounts (Mapanda et al., 2010; Mahmood, 2010).

When industrial wastewater is used for irrigation purpose, these non-essential heavy metals are taken up by plants and become the part of food chain (Ahmad et al., 2018a). In Pakistan,
the industrial effluent and municipal wastewater are drained directly into irrigation canals, streams and rivers without any treatment and accumulate in canals, rivers and reach agricultural land (Nafees et al., 2011). Contamination of water by untreated wastewater is the main reason of increased level of non-essential heavy metals (Manzoor et al., 2006).

Wheat (*Triticum aestivum* L.) is a widespread cereal crop of the world. Wheat is a staple food in Pakistan. Wheat is a chief source of food for most of the human population in the world (Khan et al., 2016). Globally, wheat is the leading source of vegetable protein in human food, having a higher protein content than other major cereals such as maize (corn), and rice (Otokunefor & Obiukwu, 2005).

The concentration of heavy metals in wheat crop increase after long-term use of industrial wastewater irrigation, which pose serious threats to human health by entering in the food chain (Abdu et al., 2011; Ahmad et al., 2018b). Prolong intake of heavy metals exert an adverse effect on animals and human health (Dogan et al., 2014; Ugulu et al., 2016). Heavy metals enter in the food chain by consumption of contaminated food crop and cause carcinogenic and non-carcinogenic (a headache, liver disease and neurological disorders) health hazards in human when their concentration exceeds the acceptable limit (USEPA, 2000). In human, chronic intake of metals causes genotoxic, developmental, gastrointestinal, dermal, cardiovascular, hematological, neurological, respiratory, reproductive and immunological disorders (Lin et al., 2013).

This study was conducted with objectives to determine the concentration of heavy metals (Zn, Mn, Ni, and Pb) in wheat irrigated with industrial wastewater and to also calculate the bioconcentration factor for each metal.

**MATERIALS AND METHODS**

**Study area**

The current research was carried out at Department of Botany, University of Sargodha, Punjab, Pakistan. Sargodha district has extreme climatic conditions. In summer the maximum temperature goes up to 50 °C and minimum up to 12 °C in winter.

**Plant cultivation and harvesting**

A pot trial was conducted in a natural environment from November 2015 to April 2016. Firstly twelve pots were taken and filled with 4 kg of soil each. In each pot, 10 seeds of wheat variety (Chagi-4) were sown. The plants were irrigated with sugar industry effluent in different concentration: T-I; 100% groundwater, T-II; 30% industrial wastewater and 70% groundwater, T-III; 60% industrial wastewater and 40% groundwater and T-IV; 90% industrial wastewater and 10% groundwater. The industrial effluent was collected from Ramzan Sugar Mill Industry situated in District Chiniot. Drip irrigation was done twice a week.

At maturity, morphological parameters of plants were determined. Harvesting was done in April 2016. After harvesting, seeds were separated from the husk. Soil samples were taken from the upper profile of soil. Both soil and grains samples were oven dried at 72 °C and were crushed into a fine powder with the help of an electrical grinder.

**Analysis of physicochemical properties of water and soil samples**

The physicochemical properties of soil and water samples such as organic matter, electrical conductivity (EC), pH, calcium, magnesium, chloride and available P were determined. Electrical conductivity was determined by a method described by Richard (1954). pH of samples was determined by pH meter (Mclean, 1982). Titration method was used for determination of Ca$^{2+}$ and Mg$^{2+}$ and Cl$^-$. Organic matter of soil was determined by Walkley and Black acid digestion method (Page, 1982). Available P and K were determined by following Olsen & Sommers (1982).

**Digestion of soil and grain samples**

The soil and grain samples (each 1 g) were processed with 15 mL mixture of HNO$_3$, HClO$_4$, and H$_2$SO$_4$ in 5:1:1 at 80°C for 2h until the digestion solution became colourless. Filtered the digest and diluted it with distilled water to make 50 mL volume (Allen et al., 1986).

**Digestion of water samples**

Digestion of Sugar industry wastewater and ground water was done by method described by APHA (2005). 10 mL con. HNO$_3$ and 50 mL water sample was taken in a beaker and maintained it on a hot plate at 80°C. When mixture was reduced to 20 mL added more 5 mL HNO$_3$ and kept on heating until transparent solution was obtained. Filtration was done by Whatman filter paper # 42 and made 50 mL volume by adding distilled water.
Metal analysis

Determination of metals in digested samples was done by using Atomic Absorption Spectrophotometer (AA-6300 Shimadzu Japan). Standard calibration curve was drawn for each metal. The metal under investigated was manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn). Instrument operating conditions for these metals were given in Table 1.

<table>
<thead>
<tr>
<th>Element</th>
<th>Mn</th>
<th>Ni</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wave length (nm)</td>
<td>238.3</td>
<td>316.8</td>
<td>198.8</td>
<td>314.9</td>
</tr>
<tr>
<td>Slit width (nm)</td>
<td>0.2</td>
<td>0.7</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Lamp current low (mA)</td>
<td>12</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Air flow rate (L/min)</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Acetylene flow rate (L/min)</td>
<td>2.2</td>
<td>1.8</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>Burner height (mm)</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Oxidant (Fuel)</td>
<td>Air, C2H2</td>
<td>Air, C2H2</td>
<td>Air, C2H2</td>
<td>Air, C2H2</td>
</tr>
</tbody>
</table>

Statistical analysis

One-way analysis of variance was applied for water, soil and wheat grains by using SPSS package SPSS-20. Correlation between soil and wheat grains with respect to each metal was also worked out. The differences between mean values of each soil and grain metals were determined using the Least Significance Difference (LSD) test at 0.05, 0.01 and 0.001 probability levels by following Steel & Torrie (1980).

Bioconcentration factor

To evaluate the transfer of metals from soil to edible parts of plants, the bioconcentration factor (BCF) was determined by following equation described by Cui et al. (2004).

\[
\text{BCF} = \frac{\text{metal level (mg/kg) in wheat grains}}{\text{metal level (mg/kg) in soil}}
\]

RESULTS

Morphological parameters

Industrial wastewater had a great effect on morphological characters i.e. plant height, leaf area per plant, leaf length, shoot and spike length of the wheat plant. The results showed that in all treatments non-significant effect (p>0.05) were seen on plant height, leaf area, leaf length, shoot and spike length of wastewater irrigated wheat plants. The highest values for morphological parameters were seen when T-II was applied which consisted of (70% groundwater and 30% industrial wastewater) while minimum values were obtained in T-IV, it consisted of 90% industrial wastewater and 10% groundwater (Table 2).

Table 2: Mean values of morphological parameters of Triticum aestivum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant height (cm)</th>
<th>Spike length (cm)</th>
<th>Shoot length (cm)</th>
<th>Leaf area (cm²)</th>
<th>Leaf length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-I</td>
<td>4.533±0.66</td>
<td>8.100±0.88</td>
<td>4.300±0.56</td>
<td>6.057±0.33</td>
<td>1.263±0.17</td>
</tr>
<tr>
<td>T-II</td>
<td>5.066±0.99</td>
<td>1.030±0.55</td>
<td>4.866±0.77</td>
<td>7.227±0.89</td>
<td>1.243±0.55</td>
</tr>
<tr>
<td>T-III</td>
<td>3.733±0.09</td>
<td>8.600±0.66</td>
<td>3.666±0.16</td>
<td>3.037±0.16</td>
<td>1.090±0.18</td>
</tr>
<tr>
<td>T-IV</td>
<td>3.433±0.77</td>
<td>6.500±0.99</td>
<td>3.133±0.08</td>
<td>2.875±0.99</td>
<td>1.316±0.12</td>
</tr>
</tbody>
</table>

Results of water, soil and wheat grains

The results from the analysis of variance of the data exhibited non-significant effect (p>0.05) of treatments on Zn and Pb in water, Mn and Zn in soil and Mn and Ni in grains, while significant effect (p<0.05) was observed for Ni, and Mn in water, Ni and Pb in soil and Pb and Zn in grains (Table 3).

Table 3: Analysis of variance of heavy metals in water, soil and wheat grains

<table>
<thead>
<tr>
<th>Metal</th>
<th>Mean Square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn</td>
</tr>
<tr>
<td>Water</td>
<td>0.076**</td>
</tr>
<tr>
<td>Soil</td>
<td>0.060**</td>
</tr>
<tr>
<td>Grains</td>
<td>0.032**</td>
</tr>
</tbody>
</table>

* , ***, Significant at 0.05, 0.001 level, ns: non–significant

Physicochemical parameters of water

In water, EC ranged from 5.1 to 8.2 with mean concentration of: T-I - 7.0, T-II - 5.14, T-III - 6.2, T-IV - 8.2 dS/m. The value of Ca²⁺ and Mg²⁺ among four treatments were 7.8, 7.6, 7.9, 8.2 mg/L, respectively. The value of CI⁻ varied from 1.11 to 1.176 mg/L with mean concentrations of T-I - 1.11, T-II - 1.26, T-III - 1.22, T-IV - 1.76 mg/L, respectively (Table 4).
Table 4: Physico-chemical parameters of irrigation water

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Physico-chemical parameters</th>
<th>EC (dS/m)</th>
<th>Ca²⁺+Mg²⁺ (mg/L)</th>
<th>Na⁺ (mg/L)</th>
<th>Cl⁻ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-I</td>
<td></td>
<td>7.96</td>
<td>7.88</td>
<td>0.16</td>
<td>1.11</td>
</tr>
<tr>
<td>T-II</td>
<td></td>
<td>5.14</td>
<td>7.66</td>
<td>0.14</td>
<td>1.26</td>
</tr>
<tr>
<td>T-III</td>
<td></td>
<td>6.21</td>
<td>7.93</td>
<td>0.15</td>
<td>1.22</td>
</tr>
<tr>
<td>T-IV</td>
<td></td>
<td>8.20</td>
<td>8.25</td>
<td>0.24</td>
<td>1.76</td>
</tr>
<tr>
<td>Standards limits</td>
<td></td>
<td>5.1⁸</td>
<td>200⁰, 150⁰</td>
<td>900⁰</td>
<td>–</td>
</tr>
</tbody>
</table>

Sources: ⁸MWE (2005), ⁰FAO (1985)

Physico-chemical parameters of soil

The soil in four treatments was clay loam. The mean values of pH ranged from 8.0-8.1. The range of EC in four treatments was found between 1.50-1.80 dS/m with a mean concentration of T-I - 1.80, T-II - 1.58, T-III - 1.58 and T-IV - 1.80 dS/m. The percentage of organic matter among four treatments ranged from 1.11 to 1.35. The mean values of available P were 2.91, 1.72, 1.71 and 1.90 mg/kg in T-I, T-II, T-III and T-IV, respectively. The values of available K in all treatments were 5.22, 4.90, 3.40 and 5.90 mg/kg in T-I, T-II, T-III and T-IV, respectively (Table 5).

Table 5: Physico-chemical parameters of soil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>EC (dS/m)</th>
<th>Organic matter (%)</th>
<th>Available phosphorous (mg/kg)</th>
<th>Available potassium (mg/kg)</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-I</td>
<td>8.1</td>
<td>1.80</td>
<td>1.25</td>
<td>2.91</td>
<td>5.22</td>
<td>Loam</td>
</tr>
<tr>
<td>T-II</td>
<td>8.2</td>
<td>1.58</td>
<td>1.11</td>
<td>1.72</td>
<td>4.90</td>
<td>Loam</td>
</tr>
<tr>
<td>T-III</td>
<td>8.3</td>
<td>1.58</td>
<td>1.18</td>
<td>1.71</td>
<td>3.40</td>
<td>Loam</td>
</tr>
<tr>
<td>T-IV</td>
<td>8.2</td>
<td>7.89</td>
<td>1.50</td>
<td>1.90</td>
<td>5.90</td>
<td>Loam</td>
</tr>
</tbody>
</table>

Heavy metal concentrations in water

The range of heavy metals in water of four treatments was: Mn (0.29-0.61), Ni (0.79-1.02), Pb (0.01-0.42) and Zn (0.61-0.80). The decreasing order of heavy metals at T-I, T-II and T-III was: Pb<Mn<Zn<Ni, while in T-IV it was found in the following sequence: Pb<Mn<Ni<Zn. (Table 6, Figure 1).

Table 6: Mean concentrations of heavy metals in irrigation water, soil, in grains of *Triticum aestivum*

<table>
<thead>
<tr>
<th>Metal</th>
<th>T-I</th>
<th>T-II</th>
<th>T-III</th>
<th>T-IV</th>
<th>Permissible limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>0.291±0.04</td>
<td>0.319±0.04</td>
<td>0.403±0.05</td>
<td>0.611±0.08</td>
<td>0.2⁰</td>
</tr>
<tr>
<td>Ni</td>
<td>0.856±0.04</td>
<td>0.893±0.05</td>
<td>1.020±0.06</td>
<td>0.793±0.05</td>
<td>0.2⁰</td>
</tr>
<tr>
<td>Pb</td>
<td>0.013±0.03</td>
<td>0.017±0.02</td>
<td>0.219±0.05</td>
<td>0.423±0.04</td>
<td>0.1⁰</td>
</tr>
<tr>
<td>Zn</td>
<td>0.678±0.05</td>
<td>0.66±0.04</td>
<td>0.615±0.07</td>
<td>0.803±0.07</td>
<td>2⁰</td>
</tr>
<tr>
<td>Soil (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>0.723±0.09</td>
<td>0.403±0.1</td>
<td>0.483±0.11</td>
<td>0.611±0.9</td>
<td>46.74⁰</td>
</tr>
<tr>
<td>Ni</td>
<td>1.137±0.08</td>
<td>1.140±0.1</td>
<td>1.118±0.12</td>
<td>1.138±0.11</td>
<td>9.06⁰</td>
</tr>
<tr>
<td>Pb</td>
<td>0.068±0.07</td>
<td>0.260±0.9</td>
<td>0.376±0.08</td>
<td>0.736±0.12</td>
<td>350⁰</td>
</tr>
<tr>
<td>Zn</td>
<td>0.393±0.05</td>
<td>0.180±0.08</td>
<td>0.139±0.09</td>
<td>0.396±0.08</td>
<td>44.19⁰</td>
</tr>
<tr>
<td>Grains (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>0.750±0.11</td>
<td>0.774±0.09</td>
<td>0.836±0.11</td>
<td>0.981±0.09</td>
<td>500⁰</td>
</tr>
<tr>
<td>Ni</td>
<td>0.346±0.09</td>
<td>0.321±0.08</td>
<td>0.641±0.1</td>
<td>0.736±0.1</td>
<td>67⁰</td>
</tr>
<tr>
<td>Pb</td>
<td>0.183±0.08</td>
<td>0.385±0.02</td>
<td>0.195±0.3</td>
<td>0.524±0.11</td>
<td>0.3⁰</td>
</tr>
<tr>
<td>Zn</td>
<td>0.183±0.07</td>
<td>0.385±0.03</td>
<td>0.195±0.07</td>
<td>0.524±0.4</td>
<td>99.4⁰</td>
</tr>
</tbody>
</table>


Fig. 1: The variation of heavy metals in irrigation water
**Heavy metal concentrations in soil**

The range of heavy metals noticed in soil was: Mn (0.40-0.72), Ni (1.11-1.14), Pb (0.06-0.73) and Zn (0.13-0.39) mg/kg, respectively. The trend of heavy metals in T-I was Pb<Zn<Mn<Ni, T-II and T-III was Zn<Pb<Mn<Ni. While in T-IV it was found in the following sequence: Zn<Mn<Pb<Ni. Values of Zn was higher while Ni was lower in all treatments (Table 6, Figure 2).

![Fig. 2: The variation of heavy metals in soil](image)

**Heavy metal concentrations in grains**

The range of heavy metals in all treatments was: for Mn 0.18-0.75, for Ni 0.32-0.77, for Pb 0.19-0.83, for Zn 0.52-0.98, respectively. The order of concentration of heavy metals in T-I - T-III and T-IV was: Pb, Zn<Ni<Mn. While in T-II it was found in increasing order of: Ni<Pb<Zn<Mn (Table 6, Figure 3).

![Fig. 3: The variation of heavy metals in grains of *Triticum aestivum*](image)

**Correlation**

In present study, Mn, Ni and Zn showed positive and non-significant correlation between soil and grains of wheat (Table 7).

**Table 7: Correlation between soil and grain metal concentrations**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Soil-grains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>0.364&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Ni</td>
<td>0.079&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Pb</td>
<td>-0.38&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Zn</td>
<td>0.19&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

ns: non-significant

**Bioconcentration factor**

The order of BCF in T-I was: Pb>Mn>Ni>Zn, in T-II: Zn>Mn>Pb>Ni, in T-III: Mn>Zn>Ni>Pb, while at T-IV it was found in increasing order of: Mn>Zn>Pb>Ni (Table 8). Highest BCF was obtained for Zn and Mn.

**Table 8: Bioconcentration factor for soil-plant**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heavy metal concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn</td>
</tr>
<tr>
<td>T-I</td>
<td>1.037344</td>
</tr>
<tr>
<td>T-II</td>
<td>1.920596</td>
</tr>
<tr>
<td>T-III</td>
<td>1.730849</td>
</tr>
<tr>
<td>T-IV</td>
<td>1.605565</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Industrial wastewater had a great effect on morphological characters i.e. plant height, leaf area per plant, leaf length, shoot and spike length of the wheat plant. These morphological characters were higher in control treatment and decreased with increased concentration of industrial wastewater. A similar reduction in different morphological parameters was examined by Vijayaragavan et al. (2006). Present results for leaf area, leaf length, root and shoot lengths were found similar to the findings of Metwalli et al. (2013). Growth and germination inhibited by higher concentration of waste water. Waste water is one of the major factors behind low productivity of crops as reported by Konwar & Jha (2010).

Ionic concentration of water is determined by calculating its electrical conductivity. The values...
of EC for Cl, Ca$^{+2}$, and Mg$^{+2}$ in current investigation were lower than the findings of Nafees & Amin (2014). Alghobar et al., (2014) reported higher values for these parameters as compared to present results. It was found that by increasing concentration of industrial wastewater to level of Ca$^{+2}$, Mg$^{+2}$, and Cl$^-$ was also increased.

Soil pH decreased by application of industrial wastewater as compared to control. Similar results were found by Li et al., (2001) who also reported decrease in soil pH due to wastewater irrigation. Decrease in soil pH may be due to the decomposition of organic matter present in soil (Vaseghi et al., 2005). Wastewater irrigation considerably increases the EC, organic matter, available P, and available K. Our results are similar to the findings of Alghobar et al. (2014) and Verma et al. (2015).

According to FAO (1985) and WWF (2007) values of Pb, Mn, and Ni are above the permissible limit except for Zn, whose values fell within the permissible limit. Present concentrations of Pb and Zn were lower while Mn and Ni were higher than those recorded by Hassan et al. (2015). The concentration of heavy metal increased by increasing dose of sugar industrial effluent on agricultural land.

Present values for all metals were below the maximum permissible limits given by CSEPA (1995) and Singh et al. (2010). So the level of these metals in soil samples was found within safe limits.

In current results, concentrations of all metals were lower than the values reported by Alghobar et al. (2014). Khan et al. (2013) also reported higher values for these metals in their work. It revealed that these metals were not properly translocated to crop. Present values for all metals were below the maximum permissible limits given by FAO/WHO (2001) except for Pb. The permissible limit for lead is 0.3 mg/kg given by FAO/WHO (2001). The result obtained in this investigation was higher than the recommended level. Lead is a toxic heavy metal and causes physiological, hematological and neurological disorders (Sorme & Lagerkvist, 2002).

Pb showed negative and non-significant results between soil and wheat grains. In present investigation, Mn, Ni and Zn showed positive and non-significant relationship between soil and grains. Negative non-significant correlation for Pb indicated weak relationship between soil and wheat grains. Positive and non-significant correlation for various metals was also observed by Khan et al. (2013).

Bioconcentration factor is an important parameter to determine the extent of metal transfer from soil to eatable parts of plant. Present BCF value for all metals was higher as compared to BCF values for different metals like Mn, Ni, Pb, and Zn reported by Jaishree & Khan (2015). In present study, Pb and Zn showed higher BCF than those reported by Asdeo et al. (2014). The present concentration of Zn and Ni were also higher than those reported of Verma et al., (2015). Bioconcentration factor was higher for Zn and Mn indicating that these metals had high tendency to move from soil to crop. Pb and Ni had low mobility as compared to other metals.

**CONCLUSION**

Use of industrial effluent and municipal wastewater in agriculture has increased due to the scarcity of fresh water resources. Industrial wastewater is contaminated with heavy metals and poses serious threats to the sustainability of ecosystem mainly human beings. Industrial wastewater irrigation considerably increased the EC for Ca$^{+2}$, Mg$^{+2}$, Cl$^-$, available P, and available K of soil. Variation of heavy metals concentration in grains samples showed the difference in uptake ability of these metals by wheat. Level of all heavy metals in our findings fell within tolerable range except for Pb whose concentration surpassed the acceptable limit recommended by FAO/WHO. Absorption of Pb by consumption of wheat grains poses serious threats to the lives of humankind. So, it is recommended that industrial wastewater should be treated properly before its application on agricultural land to reduce the extent of metal contamination in soil and wheat crop.

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Investigation of Nickel in soil, forages and blood plasma of buffaloes with respect to seasonal variations

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INTRODUCTION
Various kinds of pesticides, polycyclic aromatic hydrocarbons, heavy metals, inorganic fertilizers and other such pollutants contaminate the environment (Gong et al., 2004) These pollutants are emitted from various sources like Agriculture, commercial and vehicular emission, industries etc. (Srivastava, 2001). Among the worst sources of the pollution are the anthropogenic activities like fuel combustion, construction, traffic and waste disposal etc. Although that industrial areas are moved away from the residential areas but the road side traffic and railway terminals still remains the sources of pollution. Heavy metals like Zn, Fe and Ni are also
released from the components of pipe, alloy, tyre in the motor vehicles. These heavy metals enter into the soil and get accumulated. Not only the soil fauna and flora are affected by the elevated concentrations of the heavy metals but they also affect the forages, vegetables and fruits etc. These heavy metals leach down to the underground water through run off. The heavy metals released into environment by vehicles are dispersed by rainfall or by wind and thus they enter into the soil ecosystem. Thus in order to check the pollution caused by vehicles, the chemical and biological analysis of the soil samples may be performed in order to estimate the extent of pollution.

Among the heavy metals, nickel as component of important enzymes urease was discovered in 1975 (Taksihima et al., 1998). The nickel is an important part of the urease enzyme required for nitrogen metabolism and it is also an essential nutrient for the growth of plants. But the nickel is phytotoxic in large amount. The nickel toxicity appears as growth inhibition, disturb sugar transport, necrosis, chlorosis, wilting and affect seed germination. The pollution due to Nickel per year is almost 150,000 and 180,000 metrics tons. The sources of nickel entrance in the environment are mostly roadside traffic emissions, industrial production and fossil fuel consumption etc. The disturbed nitrogen metabolism affects the growth and reproduction in plants etc. The concentration of nickel in polluted soil (26000 ppm) is about 20-30 times greater than the nickel permissible limit. Nickel is also found to disturb the uptake of phosphorus by plants.

With the increase in the population day by day, urbanization and industrialization had become a serious issue. Organisms have to eat polluted feeds and they are helpless to live in a highly contaminated environment. The animal feeding areas close to the roads are mainly subjected to the heavy metal contamination. Heavy metals after getting entry into food chain are found responsible for various disorders (Makridis & Daras, 2012). The need of hour is that the heavy metals concentration of the road side forages, soil and the blood of animals like buffalo rearing on these forages should be analyzed. This study aims at determining of Ni concentrations in the automobile contaminated soil, forage and blood of buffalo so that some precautionary measure could be adopted.

MATERIALS AND METHODS

Study area

The area selected for study was Sargodha, which is the 11th most populated metropolitan city in Pakistan, is located between 32°3” E and 32°7”E and between N and 72°38”. This city of Pakistan has hot and cold climate. Flat surface of Sargodha has an altitude of 190m (Topo Contour, 2015). The Sargodha city has an area of about 52km² and is having population of 0.7 million. Major parts of a country are connected with Sargodha by road and rail.

This study was done on roadside contaminated forages, soil and buffalo blood of various areas of Sargodha city. The six different roadsides of Sargodha were selected to collect the samples of forages, soil and buffalo blood serum. The sampling for this study was done in two seasons’ winter and summer in December 2016, January 2016 and May 2016, June 2016, respectively.

COLLECTION OF SAMPLES

Forage and soil samples

The roadsides selected for sampling were 50 Chak, Shaheenabad, Bhalwal, Faisalabad, and Mateela. The site away from the road was Dera Saudi. 120 samples of forage and soil samples were collected in winter and summer. The two g samples of each forage and soil were kept in plastic bags after their air and oven drying (at 70-75°C for 7 days).

Blood samples

The samples of the Buffalo blood were collected by sterilized needles from the vein (jugular) of Buffalo. The blood was protected from clotting by placing them in heparinized Na-citrate vials. The blood serum samples were centrifuged for 15-30min at 3000rpm. After centrifugation the samples of serum were placed in labeled vials in a freezer at 20°C.

Forage

125 ml conical flask was taken in to which about 0.5g of the sample of forage was added. Along with this 25ml of concentrated (55%), 5ml of concentrated (72%) hydrochloric acid (HCl), 5ml of concentration (98%) sulphuric acid (H₂SO₄) were also added to this flask in order to digest the sample. This whole solution was heated till the appearance of white fumes.

Soil

The soil samples were digested by utilizing 1g of the soil samples adding 1ml of per chloric acid and 5ml of concentrated trioxonitrate acid. This mixture was added to the digestion flask, This mixture was heated up to 80-90°C until the white fumes started to appear (Allen et al., 1976). The above solution was cooled and diluted up to 50ml.
Blood samples were digested using concentrated nitric acid and perchloric acid. The 0.5 g sample of blood serum was digested by using 10ml of nitric acid and 5ml of perchloric acid in a digestion flask. Heating of this solution resulted in a clear solution which is our desired digested sample (Richards, 1968).

Dilution and filtration
All the digested samples were diluted by freshly prepared distilled water making their volume up to 50ml. The dilution of the samples was followed by their filtration and labeling. All the samples were then reserved in plastic bottles.

Formation of standard solution for heavy metal analysis
To standardize the atomic absorption spectrophotometer to find the precise value, it was needed that standard solution of the heavy metals (Ni, Cd, Cu, Fe, Cd, Cr, Co) to be analyzed be prepared.

Instrumentation
The instrumentation used in this study was the atomic absorption spectrophotometer Model AA-6300 which was used to analyze the heavy metals in the digested samples. This technique involves the measurement of the absorbed radiations by the heavy metal in the sample. The extent up to which the radiations are absorbed is estimated by reading the spectra produced on sample excitation. This instrument follows the Beer-Lambert Law.

Statistical analysis
SPSS software (version 20) and Two-way ANOVA were the used to find the variance for the concentration of heavy metals in soil, forage and buffalo blood serum. Statistical significance between the mean was tested at 0.05, 0.01 and 0.001 level of probability as suggested by Steel & Torrie (1980).

Bio-concentration factor
Bio-concentration factor formula was calculated by the following equation according to Smith et al. (2013)

\[
\text{BCF} = \frac{\text{Content of heavy metal in forage}}{\text{Content of heavy metal in corresponding soil}} \text{ (mg kg}^{-1}\text{)}
\]

Daily intake of metal
The estimated daily intake dose (EDI) was calculated as follows:

\[
\text{EDI} = \frac{C_i \times \text{IR}}{\text{BW}}
\]

In this equation BW (kg) stands for the body weight of buffalo (550 kg per cattle (Briggs & Briggs 1980), IR (g per day) the average daily consumption of forages by buffalos which is 12.5 kg where CI (mg kg\(^{-1}\)) is the concentration of heavy Ni in forages.

Health risk index
The formula given below (USEPA, 2002) was used to find health risk index:

\[
\text{HRI} = \frac{\text{DIM}}{\text{RfD}}
\]

From the integrated risk information system, RfD values for Ni was 0.02 mg/kg/day (USEPA, 2010).

Pollution load index (PLI)
In order to find out the heavy metal concentration in soil samples the Pollution load index was calculated. The formula of the Pollution load index was calculated by formula given by Liu et al. (2005).

RESULTS
Analysis of soil
Nickel affected non-significantly to the sites, seasons and sites x seasons, respectively (Table 1). The concentration of Nickel in the six sites of sampling was of the order: Site II>Site V>Site III>Site VI>Site IV>Site I respectively (Table 2, Figure 1).

<table>
<thead>
<tr>
<th>Degree of freedom</th>
<th>Source of Variation</th>
<th>Mean Squares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ni</td>
<td></td>
</tr>
<tr>
<td>Sites</td>
<td>5</td>
<td>1.644(^{ns})</td>
</tr>
<tr>
<td>Seasons</td>
<td>1</td>
<td>7.84(^{ns})</td>
</tr>
<tr>
<td>Sites x seasons</td>
<td>5</td>
<td>3.41(^{ns})</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>1.393</td>
</tr>
</tbody>
</table>

\(^{ns}\) = non-significant, significant at 0.05 = *, significant at 0.01 = ** Significant at 0.001 = ***.
Analysis of forage
Sites affected non-significantly while Seasons and Sites × Seasons affected significantly on the concentration of Ni (Table 2). The concentration of Nickel in the six sites of sampling was of the order: Site II > Site III > Site V > Site VI > Site IV > Site I (Figure 2).

Reported that the difference of heavy metal concentrations in forages depend on the type of soil, the fertility of soil, the pH and the type of forage being studied (Huston et al., 2006). The lower concentrations of the heavy meals in forage sample may be due to lesser automobile exhaust as compared to the larger cities. Heavy metal levels studied by John et al. (2013) showed higher concentration of Cd, Cr, Cu and Zn as compared to that found in this study. On the other hand the concentration of Ni was found lowest as found by Bahadur et al. (2011). According to Word et al. (1977) it was reported that the accumulation of Ni along with other heavy metals in soils is caused by the emission of dangerous and poisonous gases from the vehicles.

Table 2: Analysis of variance for Nickel in forage at six sites of sampling

<table>
<thead>
<tr>
<th>Degree of freedom</th>
<th>Source of Variation</th>
<th>Mean Squares Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sites</td>
<td>5</td>
<td>11.70&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Seasons</td>
<td>1</td>
<td>0.001&lt;sub&gt;***&lt;/sub&gt;</td>
</tr>
<tr>
<td>Sites × seasons</td>
<td>5</td>
<td>0.029&lt;sub&gt;**&lt;/sub&gt;</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>0.801</td>
</tr>
</tbody>
</table>

Significant at 0.05=*, significant at 0.01=**, Significant at 0.001=***, and ns=non-significant

Analysis of blood
Site and Season affected non-significantly on the concentration of Ni while Sites × Seasons affected non-significantly on the concentration of Ni (Table 3). Concentration of Ni was found higher at Site II and lowest at Site I (Figure 3). The nickel concentration in the study done by Nwede et al., (2010) was Ni-0.41mg which was higher unlike the concentrations of Ni in current study.

Table: 3 Analysis of variance for Nickle in buffalo Blood at six sites of sampling

<table>
<thead>
<tr>
<th>Degree of freedom</th>
<th>Source of Variation</th>
<th>Mean Squares Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sites</td>
<td>5</td>
<td>4.15&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Seasons</td>
<td>1</td>
<td>39.78&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Sites × seasons</td>
<td>5</td>
<td>3.454</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>2.088</td>
</tr>
</tbody>
</table>

Significant at 0.001=***, significant at 0.01=**, Significant at 0.05=* and ns=non-significant
Bio-concentration factor for forage to blood

The Bio concentration factor for blood and forage at the six sites of sampling was of the order: Site VI>Site IV>Site V>Site II>Site III>Site I (Table 4).

The soil properties like its pH influence the mobility of heavy metals in soil. It may be possible that the pH of examined soil affect the bioconcentration factor of heavy metal. According to Liu et al. (2006) the bioconcentration factor if found greater than 1 suggest that the plants can accumulate heavy metals in them. Alloway & Ayres, 1997 reported that the extent of the heavy metal uptake by forages depends upon their age, edaphic factors and the climatic factors.

Correlation

Concentration of Ni (0.324) between soil and forage was found positive non-significant while Concentration of Ni (-0.382) between forage and blood was found negative non-significant. The trend of correlation found by Chakresh et al., (2012) was different as compared to that found in this study. It was found highest for Ni. Relationship of heavy metals suggesting imbalance of the heavy metals between soils was one of the reason behind negative non-significant correlation. Effective translocation of heavy metals from soil was one of the reasons behind the positive correlation found for Ni (Amlan et al., 2012).

Daily intake of metals

The order of the DIM was: Site IV>Site I>Site II>Site III>Site VI>Site V (Table 5).

The daily intake of metal for Nickel was found higher than reported by Lente et al. (2011). In the current results the values of DIM were lower than 1 it suggests that no risk of health is associated with the consumption of such contaminated forages (Radwan & Salama (2006)).

**Table 4: Bio concentration factor from soil to forage and from forage to blood**

<table>
<thead>
<tr>
<th>Sites</th>
<th>Soil-Forage</th>
<th>Forage-Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site I</td>
<td>0.601</td>
<td>0.409</td>
</tr>
<tr>
<td>Site II</td>
<td>1.89</td>
<td>0.86</td>
</tr>
<tr>
<td>Site III</td>
<td>1.58</td>
<td>0.73</td>
</tr>
<tr>
<td>Site IV</td>
<td>0.90</td>
<td>1.32</td>
</tr>
<tr>
<td>Site V</td>
<td>1.09</td>
<td>1.105</td>
</tr>
<tr>
<td>Site VI</td>
<td>0.803</td>
<td>1.72</td>
</tr>
</tbody>
</table>

**Table 5: Daily intake of metal and health risk index via consumption of forage from six different sites of Sargodha District**

<table>
<thead>
<tr>
<th>Sites</th>
<th>DIM</th>
<th>HRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site I</td>
<td>0.05</td>
<td>1.03</td>
</tr>
<tr>
<td>Site II</td>
<td>0.048</td>
<td>2.09</td>
</tr>
<tr>
<td>Site III</td>
<td>0.043</td>
<td>2.15</td>
</tr>
<tr>
<td>Site IV</td>
<td>0.059</td>
<td>2.95</td>
</tr>
<tr>
<td>Site V</td>
<td>0.042</td>
<td>2.145</td>
</tr>
<tr>
<td>Site VI</td>
<td>0.049</td>
<td>0.138</td>
</tr>
</tbody>
</table>
Health risk index
The order of HRI for Ni was of the order: Site IV > Site III > Site V > Site II > Site I > Site VI (Table 5). The values of nickel were higher in the samples collected and examined in this study contrary to the study done by Zahra et al. (2014). The value of the health risk index if found greater than 1 means a serious health risk is associated with the consumption of this contaminated forage and vice versa. USEPA. (2002) According to Sajjad et al. (2009) if the HRI is found greater than 1 it means a serious health risk is associated with the consumption of roadside contaminated forages. Health risk index depends on the chemical composition and the physical characteristics of soil, type of forage being consumed and rate of the consumption of forages.

Pollution load index
The order of PLI for Ni was: Site V > Site VI > Site III > Site IV > Site I > Site II (Table 6). The pollution load index observed by Ahmad et al. (2014) in samples of forage and soil was lower as compared to that found in this study. Pollution load index if found greater than 1 indicate the more contamination in the examined area unlike the pollution load index less than 1 which means that area is less polluted and there is less automobile concentration on the sampling site.

Table 6: The pollution load index in soil of forage obtained from six sites of sampling

<table>
<thead>
<tr>
<th>Study sites</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site I</td>
<td>0.29</td>
</tr>
<tr>
<td>Site II</td>
<td>0.04</td>
</tr>
<tr>
<td>Site III</td>
<td>0.38</td>
</tr>
<tr>
<td>Site IV</td>
<td>0.35</td>
</tr>
<tr>
<td>Site V</td>
<td>0.45</td>
</tr>
<tr>
<td>Site VI</td>
<td>0.371</td>
</tr>
</tbody>
</table>

DISCUSSION
The mean values of Ni found in present study were higher. The elevated concentrations of heavy metals in the roadsides were due to the pollution by factories and traffic smoke found in the vicinity of sampling sites. On the other hand the lower values of heavy metal suggest lesser traffic in the area of sampling as compared to larger cities. In addition to that the concentration of heavy metals in soil depends on the type of soil. Arid and semi-arid soils have a higher average of trace elements concentration than those of temperate and boreal regions as well as the humid tropic zones (Fengxiang, 2007). The lower concentrations of the Ni in forage were due to the lesser automobile exhaust in the Sargodha as compared to larger city. Differences may be related to variations in botanical composition of selected diets, soil pH, soil fertility etc. (Huston et al., 2002). In the blood samples Ni was found higher in our study unlike that found by Nwede et al. (2011). The concentrations of heavy metals such as Ni retained in the sensitive organs of body like liver and kidneys which further leads to poisoning by them (Minervino et al., 1999). Metallurgical waste and road side smoke should be properly managed in order to be safe from their toxic effects.

Bio concentration factor
The higher bio concentration factor of heavy metals suggest low retention of metals in soil and heavy metal get readily transported into the forages while on the other hand lower bio concentration factor suggest that heavy metals are in tight bonding with the soil and they do not get transferred readily to forage. The bio concentration factor also depends upon soil pH (Zhang et al., 2007; Cui et al., 2004). The soil properties like its pH influence the mobility of heavy metals in soil. At low pH, high mobility of heavy metals was occurred (Celechovska et al., 2008). In addition to this the translocation of heavy metals was controlled by pressure of leaf transpiration and the pressure of root (Violante et al., 2010).

Pollution load index
The pollution load index greater than 1 indicated more contamination in the study site. The lower pollution load index suggests that there was less rush of industries in Sargodha. The pollution load index is the measure of assessing soil quality where a value of PLI > 1 would indicate deterioration of site quality, where values of PLI < 1 denote perfection, PLI = 1 presents that only baseline levels of pollutant present (Thomilson et al., 1980).

Correlation
The value of correlation for Ni was similar to those found by Bushra et al. 2014 (Ni-0.09). The weak relationship between soil and forage leads to positive non-significant correlation while strong relationship between soil and forage leads to non-significant correlation.

Health Risk Index
The value of health risk index for Ni was found higher as compared to that found by Zahra et al. (2014). The trend of the values of health risk
index was the same in present study as compared to that studied by Asma et al. (2015). According to USEPA (2002), if the health risk due to consumption of contaminated forage was found greater than 1 then its means there is an obvious health risk on the buffalos but if the health risk was found more than 1 than there is no health risk to Buffalo.

CONCLUSION

Nickel accumulation in the roadside forages, soil and grazing buffalos was found. Higher concentration of Ni in samples of blood forages and soil suggested nickel is readily transferred from one trophic level to another. The alarming rate of nickel is highly toxic to the living systems as it cannot be easily removed from the buffalo bodies. So, need of hour is that strategies should be applied to get rid of the nickel toxicity.

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Cis-acting regulatory elements and transcription factors as a key regulator in plant gene expression

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**ABSTRACT**

Gene expression regulation is an important molecular process for monitoring overall expression level of genes in plants. The expression of genes is regulated by certain regulatory elements including, Cis-acting regulatory elements (CRE), transcription factors (TFs) and promoters. Cis-acting elements are actually specific class of DNA binding proteins that act at particular site of DNA. Promoters are part of DNA fragment essential for transcriptional regulation of genes with certain transcription factors. These transcription factors could be useful in developmental regulation, interpretation and validation of candidate genes. This review highlights the importance of Cis-acting regulatory elements and transcription factors for regulation of gene expression. Furthermore, the use of bioinformatics approach for identification of transcription factors and putative motifs within the promoter region has gained much success for studying DNA-protein interactions. These findings promote the importance of CRE and TF inactivation of transcriptional networks for enhanced gene expression studies in plants.

**Keywords:** promoter, TF, CRE, transcriptional network

### INTRODUCTION

Understanding molecular mechanisms associated with regulatory control parameters is an important key to success. Gene expression is a useful tool to study genetic regulation and process of transcription. Expression of genes in plants is highly controlled by transcriptional regulators. Modern technologies, which are specific for genetic engineering of plants and improvement of crops have been discovered. The development of high throughput methods for stable expression of genes in plants is valuable for many viewpoints. One important approach for plants is to cope up with all the environmental changes due to stress conditions (Baena et al., 2010; Lauria et al., 2011). Growth of a plant is completely dependent on variety of such factors, which are responsible for controlling mechanism of expression and regulation.

Various molecular processes, in all living organisms, control gene regulatory networks. The binding of DNA elements with their recognition protein factors is also a controlled process under umbrella of specific genes. In plants, the overall control of genes in developmental processes has been studied previously (Ahmad et al., 2010). Genetic engineering of plants includes insertion of foreign genes in other plants by modifying traits as desired. Production of such plants with desired traits can be achieved by specialized transformation methods. Several trials are in practice for stable transformation of genes in higher plants. The stable integration of exogenous DNA in the plastid genome of a unicellular alga, Chlamydomonas reinhardtii have been reported (Boynton et al., 1988; Blowers et al., 1989; Boynton et al., 1990).

Expression and regulation of plants are dependent on several motifs and transcription factors, which play an important role as its machinery. The presence of these major elements in plant genome contributes in control of gene expression level by recording overall interactions among regulatory proteins. The function of genes can be specified and predicted by expression and action of specialized regulatory elements or motifs. The key regulators involved in the whole process of regulation are classified according to their particular structures. All structural motifs allow binding to specific sequences which are called as DNA binding domains. One of the transcription factor

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from *Arabidopsis thaliana* is AGL3, which encodes for MADS domain. These are well known member of MADS box family and plays an important role as transcription factors (Mubeen et al., 2018). Furthermore, these Cis-acting regulatory elements have linear structure comprising of nucleotide fragments of non-coding DNA (Venter & Botha, 2010).

To measure the rate of transcription and gene regulation at wide scale, several elements of promoter including transcription factors, transcription start sites, and motif plays an important role. Promoters consists of two main parts: The first part represents “core promoter”, lies within 100-250 bp around the transcription start site. The second part represents a distal part, which contains the element that regulates the spatio-temporal expression (Mubeen et al., 2016). The, identification of these factors and regulatory elements will be useful for studying the function of conserved motif within the promoter sequence. Still, due to lack of modern technologies for validation of motifs leads towards the path of limitation. The regulation of genes is also considered as an important regulatory step for building relationship among gene networks (Mazzucotelli et al., 2008).

Transcription activation and regulation of genes is completely dependent on interaction of promoters and transcription factors. Transcription factors binds at specific sites on promoter sequences known as transcription factor binding sites, and also consists of vast amount of cis-regulatory elements. These elements are helpful to understand the spatial and temporal features of promoters. Promoters are important regulatory elements essential for transcription of all genes. These are responsible for initiation of transcription and gene regulation control process. The activity of promoter is truly dependent on its size, copy number and position.

In this review, we have highlighted the core mechanism of gene expression and transcriptional regulation for identification of Cis-regulatory elements and transcription factors by using bioinformatics approach.

**Finding transcription factors and Cis-regulatory elements using bioinformatics approach**

The identification of cis-regulatory elements (CRE) enhance our understanding of gene regulation and expression process (Cai et al., 2010). CRE consists of several transcription factor binding sites which allows specific transcription factors (TFs) to recognize their best fit and start the regulatory process. Uptil now, studies related to CRE have shown only few combinations of transcription factors. However, this is really complex mechanism to understand as to how different plants share TFs and how these TFs are actively involved in regulation of plant genes. We have identified few Cis-regulatory elements in the promoter sequence of Sucrose Phosphate synthase SPS) promoter, their TFs, TFBs and some of the DNA specific binding domains.

Currently, the use of computational methods for prediction of regulatory elements of promoter region is a common approach. The promoter region of sucrose phosphate synthase gene was matched for finding transcription factors and Cis-acting regulatory motif using high throughput genome sequencing. Sucrose phosphate synthase is a key enzyme involved in conversion of fructose-6-phosphate and UDP-glucose into sucrose-6-phosphate. This is useful in biosynthesis of plants (Winter & Huber, 2003). Sucrose phosphate synthase catalyzes the first step in the synthesis of sucrose in photosynthetic tissues. The activity of SPS has been shown to be highly regulated at different transcriptional and post-transcriptional levels (Rubab et al., 2017). One of the motif from family (bHLH) of transcription factors found was as ACE (ACGTGGA) and plays an important role as a transcriptional activator involved in cell elongation. The findings are shown in Table 1 below: The presence of ACE motif was useful for understanding the expression regulation of genes involved in cell expansion and binding to G-box motif. Whereas, the bHLH plays a vital role as one of the important protein structural motif characterizing large families of transcription factors (Massar et al., 2000). Moreover, the bHLH transcription factors are important in development of cell activity. The transcription factor (AT1G68920)for ACE motif and (AT5G5187) was searched in Plant PAN database for AE-box for its relationship with other regulatory elements. The resulting transcription factor binding sequence was found at position 0619 with the following tandem repeat (CACGTG) for ACE and at position 0470 with tandem repeat (TTCCAAATGGAA) as shown in Figure 1 and 2 below.
Table 1: Cis-regulatory motifs in SPS promoter.

<table>
<thead>
<tr>
<th>Cis-regulatory element</th>
<th>Organism</th>
<th>Sequence</th>
<th>TF family</th>
<th>TF ID</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Arabidopsis thaliana</td>
<td>ACGTGGAG</td>
<td>bHLH</td>
<td>AT1G68920</td>
<td>cis-acting element involved in light responsiveness</td>
</tr>
<tr>
<td>AE-Box</td>
<td>Arabidopsis thaliana</td>
<td>AGAAACAT</td>
<td>MADS box</td>
<td>AT5G5187</td>
<td>Active part of a module for light response</td>
</tr>
<tr>
<td>ATE</td>
<td>Arabidopsis thaliana</td>
<td>CGGTCAC</td>
<td>WRKY</td>
<td>AT1G13960</td>
<td>Act as a sequence specific DNA binding protein</td>
</tr>
<tr>
<td>ATE</td>
<td>Arabidopsis thaliana</td>
<td>GGTCAC</td>
<td>WRKY</td>
<td>AT1G55600</td>
<td>DNA binding transcription factor binding protein activity</td>
</tr>
</tbody>
</table>

Fig. 1: Shows the TF binding sequence of ACE (AT1G68920) obtained from PlantPAN 2.0.

Fig. 2: Shows the TF binding sequence of AE (AT5G5187) obtained from PlantPAN 2.0.
Fig. 3: Shows TF binding sequence of ATE (AT1G13960) obtained from PlantPAN 2.0.

Fig. 4: Shows TF binding sequence of ATE (AT1G55600) obtained from PlantPAN 2.0.

Fig. 5: Shows 3 domains of specific size at different locations of WRKY.
The WRKY domain consists of 60 amino acids having conserved sequence WRKYGQK at N-terminal along with a zinc-finger motif. Many of the plant transcription factors are rich with WRKY domains, which are involved in regulation of various functions including DNA binding specificity, host defense, biosynthesis of secondary metabolites. The WRKYGQK residues give strength to N-terminal and also enables hydrophobic interactions and also provides structural stability to beta-sheet.

### Table 2:

<table>
<thead>
<tr>
<th>Domain</th>
<th>TF Family</th>
<th>Motif</th>
<th>Position: Start-End</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB-ARC</td>
<td>WRKY</td>
<td>ATE</td>
<td>157-388</td>
</tr>
<tr>
<td>LRR-3</td>
<td>WRKY</td>
<td>ATE</td>
<td>577-596</td>
</tr>
<tr>
<td>WRKY</td>
<td>WRKY</td>
<td>ATE</td>
<td>1210-1270</td>
</tr>
</tbody>
</table>

The use of promoters and transcription factors predicted specific transcription factor binding sites appears as one of the key fact in controlling expression profiles and regulatory networks. All target genes are regulated by specialized promoters attached to the upstream of gene. However, this regulation depends on spatial and temporal expression patterns. Interaction among regulatory proteins will help to control the plant gene expression level by cis-acting regulatory elements. However, the plant regulatory elements are present in the transcribed DNA strand or can be added during posttranslational modifications (Vaughn et al., 2012). The network of regulatory elements making up a complex of gene structures results after interaction among regulatory proteins called trans elements and cis-acting elements. The level of gene expression can be active or non-active or can be slow or it can shows spatial pattern. The network of transcription factors are key regulators of overall expression profiling of genes and depends on protein transport and efficiency (Li et al. 2012).

The use of promoters is useful for understanding the events within transcriptional regulatory networks. Promoters are categorised in different types: constitutive, inducible, tissue specific and synthetic. However, one of the fourth type is synthetic promoters. These are more valuable with desired characteristics for controlling gene expression. Synthetic promoters can be designed according to structure and organization of regulatory elements. (Bhullar et al., 2003; Mehrotra et al., 2011). The number of cis- regulatory elements and transcription factors in model organisms can be identified, by various bioinformatics tools (Thomas and Chiang, 2006). Further, for analysis of sequence specific motifs by transcription factors, the first and far more important task is to identify transcription factor binding site (TFB) (Carey et al., 2009).

### Future directions

Plant biotechnology involves variety of signaling pathways being regulated under the umbrella of transcriptional regulatory networks modulated by transcription factors and cis acting regulatory elements for controlling the expression of target genes. Many of the TFs are involved in regulation of diverse genes and expression patterns in Arabidopsis. Understanding the diverse expression patterns will help to explore more deeper functional studies on a transcriptional scale. Moreover, use of high-throughput data analysis techniques, it is now more easy to predict possible TFBs and TFs within a promoter sequence. With the use of modern technology approach and bioinformatics, the interaction among all transcriptional network members and regulatory proteins can be found easily. This will help to understand more details of plant molecular genomics.

### REFERENCES


transformation: foreign DNA can be stably maintained in the chromosome. The Plant Cell, 1(1), 123-132.


Antimicrobial properties of *Dalbergia*, *Brassica* and *Trifolium* honey against burn Microorganisms

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⁴Faculty of life Sciences, University of Central Punjab, Lahore, Pakistan

**ABSTRACT**

This research aimed at assessing the antimicrobial properties of *Dalbergia*, *Brassica* and *Trifolium* honey samples against microorganisms isolated from infected burned skin of patients in children hospital, Lahore, Pakistan. The isolated microorganisms were identified as *P. aeruginosa*, *E.coli*, *K. pneumoniae* and *S. aureus*. The original bacterial inoculum was serially diluted (adjusted to 1.5 X 10⁶ CFU) and spread on the nutrient agar plates. Whatmann filter paper discs were soaked in three different concentrations (50%, 70% & 90%) of each of the three unifloral honey samples for 48 hrs. The filter discs were placed on the agar plates seeded with the individual bacteria. Solitary effect of antibiotic discs (Ciprofloxacin, Imipenem, Ceftriaxone, Amikacin, and Vancomycin) and their synergistic effect were also studied. No bacterial growth showed resistance to honey at any concentration when used individually or in combination with antibiotic although bacteria showed resistance to Ciprofloxacin and Ceftriaxone. *Brassica* honey 90% was the most effective at all concentrations with a maximum of inhibition zone 11.13 mm against *P. aeruginosa* followed by 90% *Trifolium* honey with the maximum inhibition zone 10.75 mm against *P. aeruginosa*. The *Dalbergia* honey (50%) was least effective against *P. aeruginosa*, however it inhibited *S. aureus* producing inhibition zone 10.25 mm. Honey-antibiotic combination produced inhibition zone 51 mm that was much larger than the inhibition zone produced by antibiotic or honey when applied individually. Honey, whether used individually or in combination with an antibiotic, was effective against all the bacterial isolates used in this study.

**Keywords:** *Dalbergia*, *Brassica*, honey, *Trifolium*, antibiotic discs

**INTRODUCTION**

Ever since the introduction of antibiotics, the concern of antibacterial resistance has only doubled with about 70% bacterial species resistant to known antibiotics. Gram-positive species of *Staphylococcus*, *Streptococcus*, *Enterococcus* and Gram-negative species of *Klebsiella*, *E. coli*, *Proteus*, *Serratia*, *Pseudomonas*, *Acinetobacter*, *Candida*, Herpes simplex virus, *Varicella zoster* virus are the most commonly isolated, multidrug-resistant microbes of burned. Furthermore, burn infections are characterized by slow healing and scamming. The resistant bacteria causing the burn associated infections further complicate the treatment protocols. Therefore, alternate natural therapies such as honey may offer better treatment avenues compared with the modern antibiotics (Bowler, 2002).

The antimicrobial efficiency of honey against microbial infections has been known since the ancient times. The flower nectar is collected by the honeybees of genera *Apis* and *Meliponinae* and is converted into honey through enzymatic processes involving bee salivary enzymes (Iglesias et al., 2006). Honey is known to contain more...
than 180 substances including fructose (38%), glucose (31%), carotenoids, vitamins, minerals, aromatic substances, organic acids, proteins and amino acids (Szweda, 2017; Khan et al., 2012; Poonkothai et al., 2013). Of all the amino acids present in the honey, proline is the most abundant amino acid that is derived from bee salivary secretions during the conversion of nectar to honey. In addition, gluconic acid is the predominant organic acid produced by glucose oxidase (Karabagias et al., 2014). However the honey composition is variable and depends on the type of flower bee visits for collection of nectar.

An overuse or misuse of medications including anti-infective drugs results in an antibiotic resistance (Bowler, 2002). Hence, there is a dire need to explore new antibiotics that could be used against resistant untreatable infections. This is where honey offers a unique potential to contain microflora resistant to conventional antibiotics. Thus, honey has been found to be effective against more than 70 microbial species. The probable mechanism of action is through oxidation of glucose to gluconic acid by glucose oxidase, generating hydrogen peroxide (H$_2$O$_2$) as a byproduct. H$_2$O$_2$ is a known antimicrobial agent and it is only produced when honey is diluted with water activating glucose oxidase. H$_2$O$_2$ is a powerful oxidizing agent that inhibits bacterial growth and multiplication. In addition, the gluconic acid, produced in the oxidation process, lowers the honey pH 3.2-4.5 making it unsuitable for bacterial growth (Koochak et al., 2010). The low pH range is a significant antibacterial factor for diluted honey and inhibits growth of many microbes such as E. coli that grows at a minimum pH 4.3, Salmonella spp. (4.0), P. aeruginosa (4.4), S. pyogenes (4.5) etc (O’Grady et al., 1997).

The high osmolarity of honey also offers microbial growth inhibition since honey sugar molecules can ‘tie up’ water making it unavailable for the microbial growth. Bee defensin-1, also called royalsin, is produced by the hypopharangeal glands of honey bee and it exhibits antibacterial activity against Gram-positive bacteria such as Bacillus subtilis, Staphylococcus aureus. The concentration of antibacterial, defensin-1 varies with the type of honey. The normal flora composed of approximately 40 lactic acid bacterial (LAB) strains with 13 taxonomically well-defined Lactobacillus (9 spp.) and Bifidobacterium (4 spp.) species play an important role in the antibacterial activity of honey.

This symbiotic flora produces several bioactive compounds such as organic acids, hydrogen peroxide, antimicrobial peptides, antibiotics and bacteriocins. Bacteriocins are protein complexes that are highly inhibitory towards both Gram positive and Gram negative bacteria (Szweda, 2017).

The present study aims to evaluate the antimicrobial properties of the unifloral honey collected from Dalbergia, Brassica and Trifolium species. The antimicrobial activity was tested against burn microorganisms, and the degree to which these properties persist when honey is diluted. An intra-comparison of the antimicrobial characteristics of these honey samples as well as with standard antibiotics was investigated.

MATERIALS AND METHODS

Sixty patients suffering from burn infections were selected and the microbial community was taken from deep of the wound, using a sterile cotton swab. These swabs were then streaked several times, across nutrient agar plates to obtained distinctly unconnected microbial colonies. For identification of the microbes, colony morphology and biochemical tests were performed. The Dalbergia, Brassica and Trifolium honey samples were diluted with sterile water to produce 50%, 70% and 90% concentrations (wt/wt) respectively. Sterile Whattman filter paper discs (0.6 mm) were left in each of these diluted honey samples for 48 hrs under sterile conditions. Antibiotic sterile discs of ciprofloxacin (CIP), amikacin (AK), imipenem (IMP), ceftaxime (CRO) and vancomycin (VAN) were applied according to their target action against Gram-positive or Gram-negative or both types of bacteria. These antibiotic discs were also left in each of honey concentrations (50%, 70% and 90%) of the three honeys for 48 hrs and applied to the bacterial culture spread over the nutrient agar plates. These plates were incubated at 37°C for 2 hours and the zone of inhibition was measured and recorded the next day.

RESULTS

Isolation of Microorganisms

Colonies of Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae were isolated and their morphology was examined under microscope and were identified on the basis of results of biochemical tests given in table I.
Table I: Summary of the results obtained with the biochemical tests

<table>
<thead>
<tr>
<th>Biochemical Test</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia coli</em></th>
<th><em>Klebsiella pneumoniae</em></th>
<th><em>Staphylococcus aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Staining</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Motility test</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Indole test</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Citrate test</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Urease test</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Results with disc diffusion method

**Effect of Dalbergia honey**

The largest zone of inhibition at 50% concentration of *Dalbergia* honey was produced against *K. pneumoniae* with an average value of 8.00 mm while the least average inhibition zone 6.13 mm was recorded for *P. aeruginosa*. At 70% concentration, the highest inhibition zone, 8.85 mm was recorded for *S. Aureus* while the minimum inhibition zone 6.88 mm was recorded for *E. coli*. At 90% concentration of *Dalbergia* honey, the inhibition zone 10.25 mm was recorded for *S. Aureus* while *E. coli* showed a minimum inhibition zone of 7.00 mm (Table II).

Table II: Average recorded values of inhibition zone (mm) produced by *Dalbergia* honey against microorganisms used in the study.

<table>
<thead>
<tr>
<th></th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia coli</em></th>
<th><em>Klebsiella pneumoniae</em></th>
<th><em>Staphylococcus aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone of Inhibition (mm)</td>
<td>50%</td>
<td>70%</td>
<td>90%</td>
<td>50%</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>6.13±0.1</td>
<td>7.13±0.1</td>
<td>7.50±0.11</td>
<td>8.10±0.1</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>6.88±0.12</td>
<td>6.88±0.1</td>
<td>7.00±0.1</td>
<td>7.25±0.1</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>8.00±0.16</td>
<td>7.80±0.1</td>
<td>7.75±0.14</td>
<td>8.50±0.14</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>7.60±0.11</td>
<td>8.85±0.12</td>
<td>10.25±0.16</td>
<td>7.00±0.21</td>
</tr>
</tbody>
</table>

**Effect of Brassica honey**

*Brassica* honey at 50% concentration, showed the largest inhibition zone, 8.50 mm for *K. pneumonia*. This showed the superior sensitivity towards *Brassica* honey compared with other three microorganisms. The least sensitivity was shown by *S. aureus* with an inhibition zone of 7.00 mm. *P. aeruginosa* showed the largest, 9.75 mm inhibition zone at 70% concentration of *Brassica* honey and the smallest inhibition zone, 8.5 mm for *S. aureus* appeared with 70% honey concentration. At 90% concentration, the highest average inhibition zone 10.75 mm, was obtained for *K. pneumonia* and *E. coli* showed an inhibition zone 10.25 mm. The smallest, 8.63 mm average inhibition zone appeared for *S. Aureus* (Table III).

Table III: Average recorded values of inhibition zone (mm) produced by *Brassica* honey against microorganisms used in the study.

<table>
<thead>
<tr>
<th></th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia coli</em></th>
<th><em>Klebsiella pneumoniae</em></th>
<th><em>Staphylococcus aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone of Inhibition (mm)</td>
<td>50%</td>
<td>70%</td>
<td>90%</td>
<td>50%</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>8.10±0.1</td>
<td>9.75±0.13</td>
<td>11.13±0.25</td>
<td>8.63±0.2</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>7.25±0.1</td>
<td>9.00±0.1</td>
<td>10.25±0.1</td>
<td>8.63±0.2</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>8.50±0.14</td>
<td>9.25±0.11</td>
<td>10.75±0.1</td>
<td>8.63±0.2</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>7.00±0.21</td>
<td>8.50±0.1</td>
<td>8.63±0.2</td>
<td>8.63±0.2</td>
</tr>
</tbody>
</table>
Effect of *Trifolium* honey

*Trifolium* honey (50%) concentration was the most effective against *K. pneumoniae* with an average inhibition zone 7.75 mm, followed by *S. aureus* with an inhibition zone, 7.30 mm. *P. aeruginosa* produced the smallest inhibition zone, 6.05 mm, at 50% concentration of the honey. At 70%, the largest inhibition zone, 8.67 mm was produced against *S. aureus* and the least sensitivity or the smallest inhibition zone, 7.00 mm, appeared against *E. coli* was 7.65 mm. At 90% honey concentration, the inhibition zone, 10.75 was the largest against *P. aeruginosa* and the smallest inhibition zone 8.03 mm at this concentration was produced against *E. coli* (Table IV).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>50%</th>
<th>70%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>6.05±0.1</td>
<td>8.25±0.13</td>
<td>10.75±0.23</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>6.60±0.2</td>
<td>7.65±0.16</td>
<td>8.03±0.11</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>7.75±0.13</td>
<td>8.50±0.19</td>
<td>9.40±0.2</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>7.30±0.25</td>
<td>8.67±0.23</td>
<td>9.65±0.2</td>
</tr>
</tbody>
</table>

Microorganism inhibition with standard antibiotics

*P. aeruginosa* was resistant to all three antibiotics but *P. aeruginosa* was sensitive to amikacin. *E. coli* showed resistance to ciprofloxacin and ceftriaxone but it had the greatest sensitivity towards amikacin. *K. pneumonia* was not resistant to any of the antibiotics applied. It was least sensitive towards amikacin and highly sensitive towards ceftriaxone. Of the two antibiotics applied to *S. aureus*, a greater sensitivity was obtained towards imipenem than with vancomycin (Table V).

Synergistic activity of honey with antibiotics

Of all the antibiotic and honey combinations used against *P. aeruginosa*, the most effective was the combination of antibiotic amikacin with 90% *Trifolium* honey. AK+ 90% *Trifolium* produced a zone of 44 mm while the smallest zone against *P. aeruginosa* was produced by the combination of imipenem with 50% *Trifolium* honey. IMP+ 50% *Trifolium* honey produced a zone of 12.5 mm.

Against *E. coli*, CRO+ 90% *Brassica* produced the largest zone of inhibition of 36.5 mm while the smallest zone of 19 mm was produced by the combination of ciprofloxacin with 50% *Brassica* honey. Against *K. pneumonia*, AK+ 90% *Trifolium* produced the largest zone of inhibition of 42 mm while the smallest zone of 11 mm was produced by the combination of ciprofloxacin with 70% *Dalbergia* honey. Against *S. aureus*, IMP+ 90% *Trifolium* produced the largest zone of inhibition of 51 mm.

Comparison of results

A comparative study of the three honey sample showed that *Brassica* honey was the most effective at all the three concentrations (50%, 70% and 90%), against all the three microorganisms. *Trifolium* honey was the second best effective honey at all of its three concentrations. *Dalbergia* honey used either singly or in combination with the standard antibiotics, was found to be the least effective against all the three microorganisms. The antibiotics ciprofloxacin and ceftriaxone, when used individually showed no inhibition of the bacterial isolates. However, when these antibiotics were combined with honey they produced a much larger inhibition zone. For example, *E. coli* showed resistance to ceftriaxone and highest sensitivity towards amikacin. However, the combination of CRO+90% *Brassica* honey produced 36.5 mm inhibition zone which was the largest of all the zones produced by antibiotic and honey combination against *E. coli*. In addition, this zone was also much larger than 10.25 mm zone produced by 90% *Brassica* when applied individually.
**DISCUSSION**

In the present study, *Dalbergia*, *Brassica* and *Trifolium* honey samples were investigated for their antibacterial activities and the order of antimicrobial activities was established as *Brassica* > *Trifolium* > *Dalbergia* honey.

The four bacterial isolates used in the study, *P. aeruginosa*, *E. coli*, *K. pneumonia* and *S. aureus*, were multidrug resistant tested by inhibition zone. For example, *P. aeruginosa* and *E. coli* were resistant to ciprofloxacin and ceftiraxone respectively (all of the bacterial isolates were sensitive to imipenem and amikacin). Abdallah (2016) carried out an antibacterial susceptibility test on Gram-negative bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*). Amikacin generally works through inhibition of bacterial protein synthesis by binding to 30s ribosome leading to misreading of mRNA (Ahmed et al., 2015). However, Kibret and Abera (2011), specifically experimented antimicrobial susceptibility test using *E. coli*, and showed that it was sensitive to ciprofloxacin. However, recent studies have shown that Ciprofloxacin’s sensitivity against *E. coli* declines with higher resistance patterns. The formation of these `super bacteria’ is happening because they are rapidly becoming resistant to one antibiotic after another in a very small time span. Resistance to honey has not yet been reported which makes it a very promising topical antimicrobial agent against infection of antibiotic-resistant bacteria (Dixon, 2003; Carter et al., 2016). This is likely due to the complex composition of honey, which causes the individual components to act either individually or in synergy to prevent resistance (Cooper et al., 2010).

*P. aeruginosa* is a common pathogen of infected burn wounds. Abdet al. (2007) carried out a comparative study of honey and ciprofloxacin against *P. aeruginosa* and showed that the mean inhibition zone of Sider, Acacia and Eucalyptus honey were (12.1, 11.2, 11.05 mm), respectively. These inhibition zones are comparable to 11.13 mm zone produced by 90% *Brassica* honey but *Dalbergia* and *Trifolium* produced smaller zone of 7.50 mm and 10.25 mm, respectively, against *P. aeruginosa*. Nevertheless, except for *Dalbergia* honey, the 10.75 mm zone by *Trifolium* and 11.13 mm zone by *Brassica* are significantly higher than 8 mm against *P. aeruginosa* as reported by Osman.
et al. (2003). All this work and the present study, thus, show that *P. aeruginosa* is resistant to Ciprofloxacin but not to a vast majority of honey samples (Kwakman et al., 2011). The difference in their antimicrobial activities is according to floral, botanical and geographical origins, and also to bee-origin metabolic products (Almasaudi et al., 2016).

A combination of the antimicrobial properties of clinically approved antibiotics and the antibacterial activity of honey could lead to a new spectrum of antimicrobials providing broad-spectrum coverage and consequently improving therapeutic efficiency. Alkhyaat et al. 2014 blended local Yemeni honey brands and antibiotics to compare the effectiveness of this combination with the individual honey/antibiotic against standard bacterial isolates. All diluted honey samples (25%, 50% and 75%) inhibited growth of the standard bacteria. Mixture of Gentamicin and honey samples showed maximum inhibition zones with *S. abony* and *S. aureus*, *P. aeruginosa* as 32, 30 and 16 mm, respectively. From these results, they concluded that honey could effectively complement standard antibiotics, especially in cases of pathogenic infections in general and in burn wounds in particular. Similar results were obtained in the present study using *Dalbergia, Brassica* and *Trifolium* honey. *E. coli* is resistant to Ciprofloxacin but when it was blended with *Dalbergia, Brassica* and *Trifolium* honey, inhibition zones enlarged upto 27.0 mm, which is 3-folds larger than the inhibition zones produced when honey and antibiotic were used individually. In addition, combined Imipenem and Trifolium honey (90%) produced 6-folds larger inhibition zone (51 mm) against *K. pneumonia*. This was much larger than inhibition zone 9.40 mm produced by *Trifolium* 26.60 mm produced by imipenem individually. This result argues that combination of honey and antibiotic can be more efficient especially when bacteria become resistant to antibiotics.

**CONCLUSION**

The results of this study conclude that *Brassica* honey at 50%, 70% and 90% concentrations was the most effective against the microorganisms (*P. aeruginosa, E. coli, K. pneumonliae* and *S. aureus*) followed by *Trifolium* and *Dalbergia* honey. The results also revealed that when resistant bacterial isolates were exposed to a combination of different honey samples and antibiotics, a much larger inhibition zone was exhibited. Thus, imipenem combined with *Trifolium* at 90% produced an inhibition zone 51 mm against *S. aureus* and 34.40 mm produced by imipenem, applied individually. This shows that once microbes become resistant, they can be constrained by using a combination of honey and an antibiotic.

**Conflict of Interest Statement**

All authors declare that there is no conflict of interest regarding this manuscript.

**REFERENCES**


Analysis of biological potential of Aromatic Hydrazones as Novel Therapeutic Agents

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ABSTRACT

Aromatic hydrazones and their derivatives are being explored to develop new drugs against microorganisms, resistant to presently available antimicrobials. In the present study, 13 hydrazone derivatives were screened in vitro for their biological activity against microorganisms by employing agar well diffusion method. The microbes, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were cultured on agar plates. Wells in agar plates were loaded with 50 μl of 0.02 μg/ml and 0.04 μg/ml of hydrazone derivatives. Biological activity was examined by measuring the zone of growth inhibition. Compounds, 5, 6 and 11 exhibited none or negligible zone of inhibition against *S. aureus*. Compounds, 1, 4, 7, 8, 10 and 12, showed moderate zones of inhibition. Compound, 13, was found effective against *S. aureus*. Against *E. coli*, the compounds, 5 and 8, showed no zone of inhibition; the compounds, 1, 2, 3, 4, 6, 7, 12 and 13, showed little zone of inhibition, whereas the compound 9 and 11, reflected moderate zone of inhibition. Compound, 10, was found to be the most effective. When these compounds were investigated against *C. albicans*, the compounds, 1, 2, 3, 4, 5, 7 and 10, showed little zone of inhibition; the compounds, 6, 8, 9, 11 and 12, exhibited moderate activity. Compound, 13, was found to be the most effective against *C. albicans*. Overall, hydrazone derivatives are very effective against microorganisms and should be evaluated against other microbes to determine their anti-microbial potential.

Keywords: Hydrazones, Biological activity, Agar well diffusion method, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*

INTRODUCTION

Hydrazones are biologically functional drug molecules which have appealed the scientists due to their broad spectrum pharmacological worth (Raj et al., 2016). Anti-microbials are one of the most effective weapons in fighting against bacterial diseases and have immensely contributed to the health-associated aspects of human body. Various antibiotics have either become ineffective or have significantly diminished effectiveness against infections (Kim et al., 2010; Kaplançikli et al., 2007). During the last decade, the number of fungal infections has alarmingly increased in humans. To overcome this problem, novel aromatic hydrazone derivatives are synthesized and screened against fungal pathogens (Özdemir et al., 2007). The antibacterial and anti-fungal behavior of hydrazones and their derivatives have been studied on large scale to explore new compounds as antimicrobial agents. Presently, the development of effective chemotherapeutic drugs is a challenging task and hydrazone derivatives are evaluated as effective compounds against bacterial infections (Deep et al., 2010).

The benzimidazole compounds are reported to be effective against both gram-positive and gram-negative bacterial strains because of their 5, 6 dinitro and thioalkyl or thioaryl groups...
Similarly, 2-(trifluoromethyl)-1H benzimidazole derivatives are highly effective in vitro as antiparasitic compounds against T. vaginalis, E. histolytica, T. spiralis and G. intestinalis (Hernández et al., 2015). Some derivative of hydrazones, such as trihalogen benzimidazole analogues, have displayed effective antibacterial activity against S. aureus (Tuncbilek et al., 2009). A newly synthesized phosphonoyl hydrazone compound has shown efficiently superior activity against different bacteria (Ramin et al., 2013). Likewise, synthetic N-alkyl 2 Phenyl-1H Benzimidazole-5-carboximidines compound, is effective against S. aureus and other microbes (Göker et al., 2005). It is proposed that synthetic compounds of hydrazone are antibacterial agents against four gram-positive bacteria, S.aureus, S. epidermidis, B. cereus and M. luteus, and three gram-negative bacteria, E.coli, P. aeruginosa and K. pneumoniae. They are also effective against two fungi, namely A. niger and A. fumigatus (Suman et al., 2011).

The compound, 4-fluorobenzoic acid [(5-bromothiophen-2-yl) methylene] hydrazide, has displayed the most activity against Candida albicans with great inhibitory zone. Benzimidazole and their derivatives possess anti-fungal activity. Benomyl, thiabendazole and thiophenate methyl are major examples of fungicide class as reported in the literature (Özkay et al., 2010). The derivative of hydrazones,azole and non-azole, are generally helpful in treating candida infections; however, regardless of the valuable anti-fungal activities noticed in vitro, candidemia has appeared as a fatal disease in animals (Schiaffella et al., 2005). The derivatives of hydrazones are not only effective against bacteria and fungi but these agents have shown potential against tumors. A new synthetic hydrazone compound, N-glycosyl-N’-(5-substituted phenyl-2-furoyl) hydrazide, is found to be effective against cancer and fungi (Zining et al., 2014).

In this context, the resistant infections to microbes induce deleterious impact on human health and economy of the country. The discovery of new antimicrobial agents is a major challenge for the investigators to treat the infections. In the present study, the potential of diverse novel hydrazones against fungi, and gram-positive and gram-negative bacteria was explored with the prime objective to develop these anti-microbial and anti-fungal agents for improving the quality of human life.

**MATERIALS AND METHODS**

Aromatic hydrazone and their derivatives (13 compounds) were collected and their biological activity was assessed by agar well diffusion method. Two different concentrations, 0.02 μg/ml and 0.04 μg/ml, of the compounds were prepared. In agar plates, the wells of 6mm diameter were made with sterile Pasteur pipette. The wells were sealed with 1-2 drops of molten media. *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were grown on these agar plates. The wells were filled in with 50 μl of hydrazone compounds. The plates were turned upside down and placed at 37ºC (98.6ºF) in the incubator. Following 24 to 48 hours of incubation, development of the organisms was investigated and the width of zone of growth inhibition was measured with the help of the scale. The zone of inhibition indicated the area where the growth of the microorganisms was curbed by the action of hydrazone compounds. The antimicrobial activity of these compounds was investigated against gram-positive bacteria, such as S. aureus, gram-negative bacteria, such as *E. coli*, and fungi, such as *C. albicans* by well diffusion strategy.

**RESULTS**

**Biological Activity of Hydrazones against S. aureus**

Aromatic hydrazone and their derivatives (13 compounds) were screened against S. aureus by agar well diffusion method. Among the aromatic hydrazone derivatives, compounds 5, 6 and 11, exhibited none or negligible zone of inhibition. The compounds, 1, 4, 7, 8, 10 and 12, showed moderate zones of inhibition. The compound 13 was found to be the most effective against S. aureus. Zone of inhibition produced by hydrazone compounds against S. aureus are shown in Table I.
Table 1: Zone of inhibition exhibited by Hydrazone compounds against *S. aureus* strains

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Compound Name</th>
<th>Concentration</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.02 µg/ml</td>
<td>0.04 µg/ml</td>
</tr>
<tr>
<td>1)</td>
<td>1-(4-bromobenzylidene)-2-(2,4-dinitrophenyl)hydrazine</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>2)</td>
<td>1-(4-bromobenzylidene)-2-(4-chlorophenyl)hydrazine</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>3)</td>
<td>1-(4-chlorophenyl)-2-(2,5-dimethoxybenzylidene)hydrazine</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>4)</td>
<td>3-nitro-4-((2-(4-nitrophenyl)hydrazono)methyl)phenol</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>5)</td>
<td>4-((2-(4-nitrophenyl)hydrazono)methyl)phenol</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>6)</td>
<td>1-(2,4-dinitrophenyl(phenyl)-2-(2,3,4-dimethoxybenzylidene)hydrazine</td>
<td>N.Z.</td>
<td>N.Z.</td>
</tr>
<tr>
<td>7)</td>
<td>4-((2-(4-chlorophenyl)hydrazono)methyl)phenol</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>8)</td>
<td>1-(4-chlorophenyl)-2-(diphenylmethylene)hydrazine</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>9)</td>
<td>1-(3,4-dimethoxybenzylidene)-2-(4-dinitrophenyl)hydrazine</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>10)</td>
<td>1-(2,4-dinitrophenyl)-2-(3,4,5-trimethoxybenzylidene)hydrazine</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>11)</td>
<td>1-(2,5-dimethoxybenzylidene)-2-(4-nitrophenyl)hydrazine</td>
<td>N.Z.</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 1: Comparison of antibacterial activity of aromatic hydrazone compounds against *S. aureus* at 0.02 µg/ml and 0.04 µg/ml. The zone of growth inhibition at 0.02 µg/ml and 0.04 µg/ml are shown by brown and gray color bars respectively.

**Biological activity of Hydrazones against *Escherichia coli***

Aromatic hydrazone compounds 5 and 8 showed no zone of inhibition; the compounds 1, 2, 3, 4, 6, 7, 12 and 13 showed little zone of inhibition whereas the compound 9 and 11 reflected moderate zone of inhibition. The compound 10 was found to be the most effective as shown in Table 2.
Table 2: Zone of inhibition exhibited by Hydrazone compounds against *E. coli*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Compound Name</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.02 µg/ml</td>
</tr>
<tr>
<td>1)</td>
<td>1-(4-bromobenzylidene)-2-(2,4-dinitrophenyl)hydrazine</td>
<td>4</td>
</tr>
<tr>
<td>2)</td>
<td>1-(4-bromobenzylidene)-2-(4-chlorophenyl)hydrazine</td>
<td>6</td>
</tr>
<tr>
<td>3)</td>
<td>1-(4-chlorophenyl)-2-(2,5-dimethoxybenzylidene)hydrazine</td>
<td>7</td>
</tr>
<tr>
<td>4)</td>
<td>3-nitro-4-((2-(4nitrophenyl)hydrazono)methyl)phenol</td>
<td>7</td>
</tr>
<tr>
<td>5)</td>
<td>4-(2-(4-nitrophenyl)hydrazono)methyl)phenol</td>
<td>N.Z</td>
</tr>
<tr>
<td>6)</td>
<td>1-(2,4-dinitrophenylphenyl)-2-(2,3,4-dimethoxybenzylidene)hydrazine</td>
<td>6</td>
</tr>
<tr>
<td>7)</td>
<td>4-((2-(4-chlorophenyl)hydrazono)methyl)phenol</td>
<td>6</td>
</tr>
<tr>
<td>8)</td>
<td>1-(4-chlorophenyl)-2-(diphenylmethylene)hydrazine</td>
<td>N.Z</td>
</tr>
<tr>
<td>9)</td>
<td>1-(3,4-dimethoxybenzylidene)-2-(4-dinitrophenyl)hydrazine</td>
<td>10</td>
</tr>
<tr>
<td>10)</td>
<td>1-(2,4-dinitrophenyl)-2-(3,4,5-trimethoxybenzylidene)hydrazine</td>
<td>17</td>
</tr>
<tr>
<td>11)</td>
<td>1-(2,5-dimethoxybenzylidene)-2-(4-nitrophenyl)hydrazine</td>
<td>10</td>
</tr>
<tr>
<td>12)</td>
<td>2-methoxy-5-((2-(4-nitrophenyl)hydrazono)methyl)phenyl acetate</td>
<td>5</td>
</tr>
<tr>
<td>13)</td>
<td>5-(4-chlorophenyl)hydrazono)methyl)-2-methoxyphenyl acetate</td>
<td>4</td>
</tr>
</tbody>
</table>

Bioactivity of Hydrazones against *E. coli*

Fig. 2: Comparison of antibacterial activity of aromatic hydrazone compounds against *S. aureus* at 0.02 µg/ml and 0.04 µg/ml. The zone of growth inhibition at 0.02 µg/ml and 0.04 µg/ml are shown by blue and yellow color bars respectively.

**Biological Activity of Hydrazones against *Candida albicans***

Aromatic hydrazone and their derivatives were evaluated against *C. albicans* as shown in table 3. The results revealed that the compounds 1, 2, 3, 4, 5, 7 and 10 showed little zone of inhibition, whereas the compounds 6, 8, 9, 11 and 12 exhibited moderate zone of inhibition. The compound 13 was found to be the most effective against *Candida albicans*.
Table 3: Evaluation of Hydrazone compounds against C. albican at 20µg and 40µg concentrations.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Compound Name</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>1-(4-bromobenzylidene)-2-(2,4-dinitrophenyl)hydrazine</td>
<td>5 6</td>
</tr>
<tr>
<td>2)</td>
<td>1-(4-bromobenzylidene)-2-(4-chlorophenyl)hydrazine</td>
<td>4 8</td>
</tr>
<tr>
<td>3)</td>
<td>1-(4-chlorophenyl)-2-(2,5-dimethoxybenzylidene) hydrazine</td>
<td>7 8</td>
</tr>
<tr>
<td>4)</td>
<td>3-nitro-4-((2-(4-nitrophenyl)hydrazono) methyl)phenol</td>
<td>9 8</td>
</tr>
<tr>
<td>5)</td>
<td>4-((2-(4-nitrophenyl)hydrazono) methyl)phenol</td>
<td>9 10</td>
</tr>
<tr>
<td>6)</td>
<td>1-(2,4-dinitrophenylphenyl)-2-(2,3,4-dimethoxybenzylidene) hydrazine</td>
<td>7 13</td>
</tr>
<tr>
<td>7)</td>
<td>4-((2-(4-chlorophenyl)hydrazono) methyl)phenol</td>
<td>4 11</td>
</tr>
<tr>
<td>8)</td>
<td>1-(4-chlorophenyl)-2-(diphenylmethylene)hydrazine</td>
<td>9 14</td>
</tr>
<tr>
<td>9)</td>
<td>1-(3,4-dimethoxybenzylidene)-2-(4-dinitrophenyl)hydrazine</td>
<td>9 13</td>
</tr>
<tr>
<td>10)</td>
<td>1-(2,4-dinitrophenyl)-2-(3,4,5-trimethoxybenzylidene) hydrazine</td>
<td>5 10</td>
</tr>
<tr>
<td>11)</td>
<td>1-(2,5-dimethoxybenzylidene)-2-(4-nitrophenyl)hydrazine</td>
<td>7 12</td>
</tr>
<tr>
<td>12)</td>
<td>2-methoxy-5-((2-(4-nitrophenyl)hydrazono) methyl)phenyl acetate</td>
<td>10 13</td>
</tr>
<tr>
<td>13)</td>
<td>5-((4-chlorophenyl) hydrazono) methyl)-2-methoxyphenyl acetate</td>
<td>10 16</td>
</tr>
</tbody>
</table>

DISCUSSION

Microbial infections are the main threat to health of humans and animals. Microbes are developing resistance against presently available antibiotics and treatment of infections is becoming more and more complicated. To explore new effective anti-microbial agents is a major field of interest for health care. In this context, hydrazones are analyzed on wide scale for their biological activity against different microbes. Hydrazone structure consists of two associated nitrogen atoms of different nature and a carbon-nitrogen (C-N) double bond that is linked with a lone electron pair of the terminal nitrogen atom. These structural factors are predominantly responsible for the biological characteristics of hydrazones (Kim et al., 2010; Brehme et al., 2007).

Results of the present study revealed that the derivatives, 5 and 8 respectively described as 4-((2-(4-nitrophenyl) hydrazono) methyl) phenol, and 1-(4-chlorophenyl)-2-(diphenylmethylene) hydrazine were inactive against E. coli, whereas the derivative 6, 1-(2,4-dinitrophenylphenyl) -2-(2,3,4-dimethoxybenzylidene) hydrazine, was inactive against S. aureus. This insignificant activity is most likely due to the presence of nitrophenyle and chlorophenyle.
groups. The present findings contradict with the results of Wankhede et al. (2016) who had shown that some of the hydrazones with metal complexes exhibited higher antibacterial activity. On the other hand, it has been reported that some hydrazone derivatives, such as 1, 2-benzisothiazolehydrazides were inactive against E. coli and S. aureus (Vicini et al., 2002). The present study results are in line with the report of Kodisundaram et al. (2013) who described that few derivative of azabicyclomonane showed no activity against S. aureus.

Among the hydrazone derivatives, the compounds 1, 2, 3, 4, 6, 7, 12 and 13 were less active against E. coli and compounds 1, 5, 7, 11 and 12 showed insignificant activity against S. aureus. Similarly it was also reported by Özdemir et al. (2009) that some derivatives of hydrazone exhibited trivial activity against E. coli. Some derivatives of benzothiazole displayed poor activity against E. coli and S. aureus because of their substitution of chloro and nitro group at position 2 (Balram Soni et al., 2012).

Some compounds showed moderate zone of inhibition against E. coli and S. aureus. The present study results are compatible with those of Ioana et al. (2008) who described that some of the 2-hydroxybenzamide derivatives showed moderate activity against E. coli and S. aureus. It has also been reported that few hydrazones which were synthesized from cholesterol derivatives showed moderate activity against E. coli (Loncle et al., 2004). Similarly majorlity of the hydrazone derivatives of quinaxaline showed moderate activity against E. coli because of their choloro, bromo and fluoro substituent group at the position of 3 (Suroor et al., 2009; Kumar et al., 2009).

Hydrazone compounds 10 and 13 showed significant biological activity against E. coli and S. aureus, respectively. These findings are compatible with the report of Khalid et al. (2018) who documented that some of the hydrazine derivatives containing a core of pyrazole scaffold showed significant antimicrobial activity against E. coli and S. aureus. Likewise, hydrazine derivatives containing azomethine and benzimidazole showed significant activity against E. coli and S. aureus (Narang et al., 2012; Ozdemir et al. 2007). The hydrazone compounds containing isoxazolyl group exhibited substantial activity against E. coli and S. aureus (Ramanpre et al., 2011).

The hydrazone derivatives, 1, 2, 3, 4, 5, 7, 10, were less active against C. albicans. These findings are compatible with the report of Papakonstantinou et al. (2002) who revealed that cyclopentylidene hydrazide showed weak activity against C. albicans. Few compounds of sulfonhydrazones and azabicyclomonan-9-one displayed less activity against C. albicans (Kodisundaram et al., 2013; Gündüzalp et al., 2014). Most of the compounds displayed moderate activity against C. albicans as reflected by the present study results. These findings are in line with the reports of Ajani et al. (2010) who documented that derivative of quinoxaline hydrazine showed moderate activity against C. albicans. The hydrazone compound 13 reflected significant activity against C. albicans. The hydrazone compounds synthesized from a variety of cholesterol were found significantly active against C. albican (Loncle et al., 2004). Similarly, benzothiazole derivatives showed significant activity against C. albicans because of substitution of 2,3 dimethoxy groups (Balram Soni et al., 2012). Overall, hydrazine derivatives have anti-microbial potential which should be further explored against other microorganisms.

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34:5629–5660.


Health comparison of farm-raised and wild-caught *Labeo rohita* (Cypriniformes: Cyprinidae)

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**ABSTRACT**

Freshwater fish are traditionally captured from rivers but due to their increasing demand fresh water fish culture is gaining popularity. The environmental changes in natural aquatic bodies due to ever-increasing input of pollutants in them adversely affect fish populations and also the pollutants get bio-accumulated in the aquatic food chain. The present study was undertaken to compare selected health parameters of farm-raised and wild-caught *Labeo rohita*. Twenty fish were taken from each of the two groups to perform kidney and liver enzymes profile and histological analysis. The results showed that the values concerning liver function enzymes were significantly altered in wild-caught fish as compared to farm raised fish. Also, significant difference was noted in serum creatinine and urea values between the two groups. Histology of liver and kidney tissues revealed signs of chronic damage in the wild captured fish as characterized by lymphocyte infiltration and altered morphology of functional units. It is concluded from findings of the present study that farm-raised *Labeo rohita* were healthier and safer to consume compared to the wild-caught fish.

**Keywords:** River Jhelum, *Labeo rohita*, Kidney, Liver, Histology

**INTRODUCTION**

Fish is a magnificent source of nutrients for human beings; a considerable segment of world’s population benefits from it for economical survival and health particularly in developing countries (Orr et al., 2012). Fish provides essential macronutrients and micronutrients, such as proteins, essential fatty acids, vitamins and minerals. Apart from providing food security, fisheries industry is involved in trade and revenue generation (Orr et al., 2012). Increased productivity from sustainable fisheries and aquaculture can be a driver for rural development by mitigating risks to livelihoods, contributing to income generation, employment opportunities, and consequently reducing hunger and poverty for millions of people in the developing world. Sustainable production of fisheries and aquaculture improve food security, promote economic growth and protect environmental and natural resources (Welcomme et al., 2010). For the sustainable supply of fish, it is important to avoid any disaster which can be in the form of environmental pollution, habitat disturbance or disease.

Pollution of aquatic habitats is of major concern due to input of contaminants from point and non-point sources, such as industries, agriculture, and rural and urban households. These contaminants pose danger to the survival of life in these water bodies (Benson et al., 2007), and they not only reduce the survival of fauna and production efficiency of the water body but also get accumulated in the food chain leading to the development of various diseases and abnormalities (Canbek et al., 2007).

River Jhelum is one of the affluent rivers of Indus River basin in Pakistan (Mirza et al., 2011). It is one of the largest rivers of Punjab and irrigates vast areas of eastern Pakistan. Along with other freshwater fauna, some important fishes belonging to the families *Cyprinidae* and *Channidae* inhabit this river (Mirza et al., 2011). *Labeo rohita* (a member of family *Cyprinidae*, commonly called "Rohu") is one of the most important commercial fishes of Pakistan and is extensively cultured in farms. However, the public demand and preference for wild Rohu is much more than the farmed fish and it has been observed that the local rivers are not able to cater this demand both in quantity and...
quality in the past few years. The water in Jhelum River gets contaminated by pollutants from some point and non-point sources, such as industries, household wastes and other human activities. Therefore the present study was conducted to compare selected health parameters of wild-caught *Labeo rohita* from River Jhelum with farm raised *Labeo rohita* to determine which is safer source of the fish for human consumption.

**MATERIALS AND METHODS**

**Sampling sites**

For sampling wild *Labeo rohita* in Jhelum River, Jammargal Dam site in the river was selected (Fig. 1a). This location in the river is extensively used by fishermen for capturing fishes. The farm-raised fish samples were procured from Government of the Punjab, Department of Fisheries, Manawan, Lahore, Pakistan (Fig. 1b).

**Fish samples**

Completely healthy fish (average weight, 922 ± 54 g and average length 37 ± 2 cm) collected from both the above-mentioned sources were used in this study. Approximately 25 specimens were collected from each site. After sampling, the fish specimens were brought to the laboratory for further processing following standard methods of handling and transportation to reduce the possibility of infection.

In the laboratory, the fish were kept in clean and disinfected aquaria to get them acclimated and prevent them from any stress. After acclimatization, blood was drawn from caudle peduncle using standard procedure (Lucky, 1977) and serum was separated. Thereafter, each fish was immediately dissected to obtain liver and kidney for histological studies.

**Serum evaluation**

The serum of fish was tested for liver enzymes; Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) using commercial kits (Randox AS 1267 and AL 1205). Serum urea and creatinine levels were also measured to determine kidney health using Randox kits, UR 107 and CR 510.

**Histological studies**

After dissection, the liver and kidney of each fish specimen were immediately put in the fixative (10% formalin) until further processing. These organs were processed for hematoxylin and eosin staining (H and E staining) for histological studies (as described by Gartner and Hiatt, 1997; Young and Heath, 2000).

**Statistical analysis**

The collected data were analyzed by using student’s *t*-test (Microsoft excel, 2017). *P* values were determined at 95% confidence level.

**RESULTS**

Liver function test using AST and ALT was performed on the serum of both wild-caught and farm-raised fish. The outcome was a significantly higher value (*p* < 0.0001) of AST and ALT observed in the serum of wild-caught fish as compared to farm-raised fish (figure 2a, b).
For the determination of kidney health, urea and creatinine levels were measured in the serum of wild-caught and farm-raised fish. The urea level was slightly higher in the farm-raised fish as compared to the wild-caught fish, whereas, creatinine level was higher in wild-caught fish (Fig. 3a,b).

Histological comparison of the liver and kidney of farm-raised and wild-caught fish showed significant differences. The liver of wild-caught fish had severe lymphocyte infiltration as compared to liver of farm-raised fish (Fig. 4a, b). The kidney of wild-caught fish had accumulation of lymphocytes and greatly reduced number of kidney functional units as compared to farm-raised reared fish (Fig. 5a, b).

Fig. 2: Comparison of liver enzymes (a. AST; b. ALT) in serum of Labeo rohita samples obtained from the wild and farm-raised.

Fig. 3: Comparison of kidney urea (a) and creatinine (b) levels in serum of farm-raised and wild-caught Labeo rohita.

Fig. 4: Histology of liver a. farm-raised Labeo rohita showing normal liver structural units; b. wild-caught Labeo rohita showing lymphocyte infiltration; H (Hepatocyte), HP (Hepatic portal vein), S (Sinusoids), L (Lymphocyte infiltration) 40x.
Discussion

Accumulation of pollutants in the aquatic environments is making them insecure not only for the inhabiting fauna therein but also for humans in terms of fish consumption. Fish are very sensitive to any change in their environment and are often used as bio indicators of several pollutants (Nsikak et al., 2007). In the present study, a comparison has been made concerning the health condition of an edible popular fish species, *Labeo rohita*, captured from the wild with its farm-raised synonym to determine its safety for human consumption.

River Jhelum is a tributary of Indus River and runs through various regions of Pakistan. During its course it is exposed to various sources of pollutants that deteriorate not only water quality but also pose threat to the survival of life therein. The disruption of aquatic habitats due to their exposure to toxic substances has led to declines in numbers and quantity of many species especially fish. The bioaccumulation of toxins and pollutants in aquatic animals occurs due to higher accumulation levels of these pollutants in their environment which, in turn, disturb the whole food-chain and eventually reach the bodies of human beings and play a role in development of various diseases (Giari et al., 2007).

In fish as in many other animals, liver is an important organ involved in the metabolism of lipids, proteins, carbohydrates, detoxification and storage of glycogen and vitamins (Kmiec, 2001). It has been reported in several studies that liver is the highest toxins accumulating target organ and plays a crucial role in disease development. Serum AST and ALT levels are common biomarkers of I health of liver and their high levels indicate poor liver health (Wales, 1983). The liver health was compared in this study between farm-raised and wild-caught *L. rohita*. The serum levels of AST and ALT were found to be significantly elevated in fish captured from the wild (river). This significant increase of AST and ALT levels in the field-captured *L. rohita* indicated that liver of these fish were damaged which might have been due to their continuous exposure to pollutants. Higher serum levels of these enzymes also indicate cardiac and liver damage (Wales, 1983). Higher AST values are indicative of inflammation and it is supported by the increased infiltration of lymphocytes in the histology of liver of wild fish. The liver damage signs, such as abnormal enzyme values and histology in the field-captured fish are indicative of stress, injury, infection and disease, which might be in response to high toxin contents in the environment of these fish. The higher levels of toxins might be the result of runoffs of fertilizers and heavy metals and other pollutants into the River Jhelum from point- and non-point sources.

Along with liver, the kidney of wild-caught fish was also found damaged. The histological comparison showed less number of kidney functional units, increased urinary spaces and lymphocyte infiltration in kidney of fish captured from the wild as compared to farm-raised fish. These observations indicate kidney damage which is supported by amplified levels of creatinine in the serum of wild-caught fish. Creatinine is a nitrogenous end product of metabolism and is released continuously from kidneys. The proper removal of this product indicate proper kidney functioning. Increased creatinine level is indicative of problem in kidney filtration process and decreased kidney function (Ajeniyi and Solomon, 2014). The wild captured fish kidney damage is evident from the higher values of this kidney function biomarker and this increase might have been due to higher pollutant content in the environment of wild-caught fish. Exposure to toxins affects kidney function which results in elevated levels of creatinine in serum (Sawsan et al., 2017).

Urea, another metabolic waste, also determines kidney function. In the present study, urea levels were slightly higher in fish samples obtained from the farm as compared to wild-caught. It is likely that due to higher serum urea levels, farm-raised *L. rohita* may also be suffering from
kidney damage; however, histological investigation showed no signs of damage. Serum urea level might have increased due to higher protein content in the diet, as urea is the byproduct of protein metabolism (Ajeniyi and Solomon, 2014). Serum urea level depends upon dietary protein content so farmed fish are prone to have higher urea content as they are fed protein-rich diets.

In conclusion, this study indicated that field-captured Labeo rohita from Jhelum River were suffering from chronic liver and kidney damage probably leading to decrease in its population. These damages are mainly due to poor water quality and increasing pollution levels in the fresh water bodies. Moreover, the farm-raised fish are safer and healthier for human consumption due to lesser exposure to pollutants than the wild-captured fish. It is recommended that measures should be adopted to keep the natural water bodies free of pollutants as much as possible to maintain the aquatic fauna and avoid extinction of species.

ACKNOWLEDGEMENT

Sincere gratitude is expressed to local governmental authorities at Jammargal Dam, Jhelum River, and Punjab Department of Fisheries, Manawan, Lahore, Pakistan, for their assistance in field sampling and use of their facilities.

REFERENCES

Influence of Salicylic Acid on salinity stress tolerance by seed priming and foliar application on Maize (Zea Mays)

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ABSTRACT
This study was intended to measure the impact of salicylic acid (SA) on maize under salt stress. For this purpose salinity effect on various morpho-physiological and biochemical attributes of plants was analyzed by launching a petri dish and pot experiment. The analysis spread out in a totally block design (randomized) with ten replicates for each salt (0 and 120 mM NaCl) and SA (0, 0.1, 0.5 and 1mM) treatment. The commercially available cultivar (cv. Faisal) of maize was planted in earthen pots for 15 days. After fifteen days of growth, seedlings were irrigated with saline water (120 mM NaCl) and SA was applied for 60 days simultaneously. To observe the germination, seed were also primed with same concentrations in petri dishes. After 60 days on final treatment harvesting was carried out, leaf samples were taken for analyzing biochemical attributes (protein contents, antioxidants enzyme activates). A decrease in seed germination percentage from 95.22 (at control) to 25.34% (at 120 mM salt stress) and shoot length from 86.12 (at control) to 42.36 cm (at 120 mM salt stress) was observed. Similar decreasing pattern of growth was observed in case of pot grown plants after 60 days. The results suggested that salt stress drastically reduced length of shoot and root, fresh / dry weight and leaf area and antioxidant enzyme activities while the use of 0.5 mM concentration of SA greatly made good progress in all these growth and biochemical parameters. Production of antioxidant compounds under salt stress is accelerated under the influence of SA. So it causes modifications in antioxidant compounds and hence increases salt tolerance under saline conditions.

Keywords: Antioxidant activities, Priming, Proline, Salinity, Salicylic acid.

INTRODUCTION
Salinity is basically the accumulation of dissolveable salts present in the soil (Silva and Uchida, 2000). 20% soil is salt affected in the world out of total irrigated area (Pitman and Lauchli, 2002). It has been estimated that the world over salt influenced land is 953 Mha which is approximately 7% of total land area (Abdelfattah et al., 2009). Almost 6.3 × 10⁶ hectares of irrigated area has become salt stressed to varying degrees in Pakistan (Malik and Shah, 1996). Of this salt affected land 9% in Baluchistan, 40% in Sindhi and half of it in Punjab (province) (Mian and Mirza, 1993). Salinity is viewed a main consideration in constraining plant development and crop yield, and salinization of irrigated and surrounding regions in the parched tropics and sub-tropics has not been reduced. In reality, it continues to increase bit by bit (Rus et al., 2000). As indicated by Saboora et al., (2006), it is expected that saline conditions are expanded at a rate of about 10% every year around the world. Pakistan has moderate to very increased salt levels, especially NaCl (Anon, 2001).

According to reports, saline conditions badly affect the plant (growth/development), reducing germination of seeds, growth of seedlings and activity of enzymes (Seckin et al., 2009). Salinity of soil is the real abiotic stress that has adverse impacts on yield and quality of plant mainly

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due to increase of Na\textsuperscript{+} and Cl\textsuperscript{−} ions in plant parts (Shilpi and Narendra, 2005). Salt stress is a real danger to land of agriculture area in arid as well as semiarid regions (El-Hamdaoui et al., 2003). Salinity badly affects the economy of country by reducing the production of crop which is of great value (Ahmed, 2008). Water logged conditions, salt stress and increased level of sodium ions have diminished the drainage capability of the soils resulting in reduced soil fertility, decrease in crop productivity and biodiversity reduction (Zia and Baig, 1986). It has been observed in most saline soil that NaCl is foremost salt speicie that leads to death of plant (Munns and Tester, 2008). Different plants have different levels of salt tolerance (Kumar et al., 2009) that also varies from species to species. However growth regulators, fertilizers and anti-oxidants can successfully minimize the effects of salt on salt affected plants (Janda et al., 1999; Shalata and Neuman, 2001). Reactive oxygen species (ROS) are increased due to saline conditions (Asada, 2006). Oxidative damage of cell due to ROS under salt stress leads to death of cell (Mittler, 2002). During normal growth there is a balance between ROS production and its utility which is regulated by defense system of oxidants (Hameed et al., 2011).The plants whose antioxidant enzymes are more active can withstand in salt stress (Gapinska et al., 2008). Salinity increases cellular components of plant and other contents like hydrogen peroxide, superoxide dismutase (SOD), ascorbate and peroxidase (POD) are also enhanced, however salt stress also reduces the catalase activity (CAT, Hassanein et al., 2009). Some other processes are also affected by salinity in plant body like biochemical, lipid metabolism, protein synthesis, photosynthesis and several enzymes (Parida and Das, 2005). Sodium ion toxicity and oxidative stress in plant is caused by salinity (Sairam and Sarivasta, 2002; Cuin and Shabala, 2007).

\textit{Zea mays} is an annual crop and belongs to family poaceae. From many years corn is known as staple food across the world and serves as feed crop and it is an important component of global food security (Campos et al., 2004). In world the productivity of maize is more than 780 million tons per year as compare to the wheat and rice (FAO, 2013). In Pakistan maize occupies a central position among the cereal crops (Dowswell et al., 1996) and ranks 3\textsuperscript{rd} (1st wheat and 2nd rice). In Pakistan the total cultivated area of maize is 1.02 million hectares with per annum production of maize grain is 2.96 million tons (GOP, 2007). The maize crop productivity is about 2,850 kg per hectares (Tariq and Iqbal, 2010). Maize is source of corn flakes, corn starch, syrups, alcohol, glucose and tanning material (GOP, 2007). Its grain has 10 percent protein, 4.8% oil, 58.72% starch fiber, 30% sugars and 1.7% ash (Chaudhry, 1983). Increased concentration of salts in soil has drastic impact on maize growth and yield (Fu et al., 2010).

Different techniques were used to minimize the salt stress effect including genetic engineering, tissue culture, exogenous application (Wang et al., 2003). External application of different compounds vitamins like ascorbic acid and SA increases growth of plant under saline conditions (Hassanein et al., 2009). Moreover SA is now considered as a salt stress alleviating compound (signal molecule) (Horvath et al., 2007) that may stimulate the production of ROS under salt stress thus having great importance in salt resistance (Borsani et al., 2001). Treatment of SA increases hydrogen peroxide level reducing oxidizing damage of plants (Wahid et al., 2007). Many researches on exogenous applications of SA reported that it affects several developmental, physiological and biochemical mechanisms in plants due to which resistance against salt stress and drought has increased remarkably (Tari et al., 2002). During salt stress episode it affects fruit yield and seed germination (Raskin, 1992), rate of transpiration, closing of stomata (Rai et al., 1986), permeability of membrane (Barkosky & Einhellig, 1993), photosynthesis and growth (Khodary, 2004; El-Tayeb, 2005). Salicylic acid plays important role in photosynthesis and transpiration (Arfan et al., 2007), enhances antioxidant enzymes production (Xu et al., 2008), and reduces accumulation of Na\textsuperscript{+} and Cl\textsuperscript{−} ions (Gunes et al., 2007). Exogenous application of SA increases the activities of antioxidant enzymes which reduce the impact of ROS (Erasalan et al., 2007). The present work is designed to check whether seed priming and foliar application of SA enhances the growth of maize by minimizing the effect of salt stress.

\textbf{MATERIALS AND METHODS}

\textbf{Plant material and experimental conditions}

The experiments were performed to study the effect of SA by two separate method of its application i.e., seed priming and foliar spray to overcome salinity stress in Maize. Healthy certified seeds were obtained from Pioneer seed company Sahiwal, Pakistan. The experiment was done at Nursery of University of Okara Punjab, Pakistan.
This experiment was performed in totally randomized complete block design under natural conditions from September-November 2014. Average temperature was 28±2°C with average relative humidity 64% during experiment period.

Experimental layout

Seed were soaked in tap water, SA and salt +SA overnight in beaker. Then these seed were placed in petri dishes having cotton at the base. Each petri dish had ten seeds. Five petri dishes were used for this experiment. 1st petri dish was a control, in which seeds were treated with simple tap water. In 2nd petri dishes were treated with 120 mM NaCl only. In next three petri dishes (3, 4 and 5) seeds were treated with SA having concentrations 0.1, 0.5 and 1.0 mM with 120 mM of NaCl. The seeds were grown for ten days. Then shoot/root length of seedlings were measured with scale and percentage of growth was also checked.

Pot experiment

Maize seeds were grown in earthen pots having soil mixture containing field soil, bhal and manure in the ratio of 1:1:1. The experiment was done in totally randomized fashion in three blocks. Each block consists of three replicates for each concentration. Each block consists of three replicates for each concentration. The 1st group was designated as control. It was given simple tap water through irrigation with foliar spray. Surfactant Tween-20 was used for the penetration of SA into leaf tissues. Each block was divided into five groups. Each group had three (one plant/pot) pots for each concentration. The 1st group was designated as control. It was given simple tap water through irrigation with foliar spray of distilled water. The 2nd group was irrigated with water containing 120 mM NaCl. Simple distilled water was also sprayed on leaves of plants of this group. Next three groups were treated foliarly with 0.1, 0.5 and 1.0 mM concentrations of SA with 120 mM NaCl treated through roots drenching for each concentration. Salt treatment through irrigation was given whenever required and spray was also done simultaneously. The treatment was given for sixty days and later on plants were harvested.

Physical characteristics of potting-mix

By using EC and pH meter, EC, TDS (Total dissolve solids) and pH of the potting mix was taken initially before and after the completion of experiment. From pot of each treatment soil sample (15 g) was randomly taken and mixed with little water and then left to set for 5 minutes then separation of water done. This water was used to record EC, TDS and pH.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH</th>
<th>EC (mS)</th>
<th>TDS (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.9</td>
<td>1.32</td>
<td>589</td>
</tr>
<tr>
<td>Salt (120 mM)</td>
<td>8.7</td>
<td>2.67</td>
<td>782</td>
</tr>
<tr>
<td>S+ SA (0.1 mM)</td>
<td>8.6</td>
<td>2.45</td>
<td>1092</td>
</tr>
<tr>
<td>S+ SA (0.5 mM)</td>
<td>8.8</td>
<td>3.13</td>
<td>1496</td>
</tr>
<tr>
<td>S+ SA (1.0 mM)</td>
<td>8.7</td>
<td>6.21</td>
<td>3310</td>
</tr>
</tbody>
</table>

Harvesting and data recording

The plants were harvested after 60-day of treatment of SA. The length and diameter of shoots was taken before harvesting. For biochemical tests leaf samples were also collected. Then plants were uprooted from each pot carefully and washed water and then dried by the use of blotting paper.

Growth parameters

Shoot length and diameter (cm)

By the use of measuring tape and Vernier caliper the length (cm) and diameter of each shoots was measured after 60 days of treatment. The length of roots / shoots was measured with scale.

Plant biomass (g)

The shoots and roots of plants were separated and washed with tap water to remove soil particles and residues. Then material was dried with blotting paper and fresh weights of plant shoots and roots were recorded. Leaf area was also measured. Then plant material was wrapped in aluminum foil and was dried in oven for 5 days at 65°C to get the dry mass.

Leaf area (cm²)


Biochemical analysis

Quantitative assay of protein

Fresh tissue of leaf (0.5 g) was crushed in ice-chilled pestle with mortar having 1 ml of 0.1 M phosphate buffer (pH 7.2). Polyvinyl polypyrrollidone (PVP) (0.1 g) and 0.5 % (v/v)
Triton X-100 were also added. The ratio of leaf tissue: buffer was kept at 1:2 (w/v) ratio was kept between leaf tissue and buffer. Then centrifugation of homogenate was done at 14,000 rpm for 30 minutes at 4°C. At the end supernatant was obtained and cooled at 0°C and used for quantitative estimation of proteins. Soluble protein contents estimation was done with the help of Racusen and Johnston’s Biuret method (1961).

Quantitative assay of enzymes
A fresh leaves sample (0.5 g) was taken. 1 ml of phosphate buffer 0.1 M (pH 7.2), 0.1 g polyvinyl polypyrrolidone (PVP) and 0.5 % (v/v) Triton X-100 were also added. All material was homogenized and centrifugation was done for 30 minutes at 14,000 rpm at 4°C. The supernatant was separated to store at 0°C which was used further for quantitative enzymes estimation. The method of Racusen and Foote (1965) with some modifications was used to measure activity of peroxidase. For this purpose, Guaiacol (1%), H2O2 (0.3 %) and Tris-HCl (0.1 M) solutions were prepared and the enzyme activity was estimated. Superoxide dismutase activity was estimated with the use of method suggested by Maral et al. (1977).

Quantitative assay of proline
Estimation of proline was done by the method proposed by Bates et al. (1973). Sample of leaf tissue (0.5g) was taken along with 10 ml of 3 % sulfosalicylic acid. Then material was homogenized and centrifuged of homogenate at 13000 rpm for 10 minutes at 4°C. Then in a test tube 2 ml acid ninhydrin and 2 ml of glacial acetic acid and 2ml of supernatant were mixed and allowed to stand for 1 hour at 100°C and then allowed to cool at room temperature. At the end, 4 ml of toluene was allowed to mix vigorously and then the sample was left to stand for 10 minutes for the separation of toluene and aqueous phases. Upper toluene layer was separated with care and by the use of toluene its absorbance was recorded at 520 nm.

Statistical analysis
The data was analyzed statistically using one way analysis of variance by SPSS (version 12.0.0) computer program.

Duncan multiple range test (at 0.05% probability level) was used to take the mean values.

RESULTS
Effect of seed priming with salicylic acid on seed germination, shoot/root length emerging from seeds under salt stress
Seed germination
The seed germination was gradually reduced by the application of salt to very much extent as obvious in table 1. The maximum germination was observed in control (95.22%) which was reduced under salt stress to 25.34%. But %age germination of seed increased significantly by priming with SA. In case of priming, the germination %age was maximum at 0.5 mM with saline conditions. However when the plants were subjected to a higher concentration of SA (1.0 mM) percentage of germination was decreased as compared to control. The percentage germination recorded for 0.1, 0.5 and 1.0 mM SA priming seed under salinity stress was 40.53, 76.46 and 70.10%, respectively.

Shoot length and root length
In case of length of shoot, it was maximum in control (10.15cm) but it was drastically reduced under salt stress (1.86 cm). Priming of seed significantly reduced the effect of salinity and was clear improvement in shoot length. Maximum increase in length (shoot) was observed at 0.5 mM priming of SA. Shoot length after seed priming at concentration levels 0.1, 0.5 and 1.0 mM of SA was 2.89, 5.76 and 4.33 cm, respectively. Same results were observed in case of root length. Control seeds showed maximum growth with root length of 11.47 cm which was greatly reduced (2.10cm) in seeds under 120 mM salt stress. Seed priming (with salicylic acid) reduced the adverse effects of salts and there was remarkable increase in root length. Maximum increase in length (root) was observed in 0.5 mM seed priming plant under salt stress. Priming with concentration levels 0.1, 0.5 and 1.0 mM of SA have root length 4.13, 6.76, and 5.89 cm, respectively.
Table I: Effect of SA (Salicylic acid) on maize seed priming under salt stress in terms of germination, shoot length/root length.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Germination %age</th>
<th>Shoot Length (cm)</th>
<th>Root Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.22 ± 2.00d</td>
<td>10.15 ± 2.20e</td>
<td>11.47 ± 2.28e</td>
</tr>
<tr>
<td>Salt (120 mM)</td>
<td>25.34 ± 3.50a</td>
<td>1.86 ± 0.40a</td>
<td>2.10 ± 0.55a</td>
</tr>
<tr>
<td>S + SA (0.1 mM)</td>
<td>40.53 ± 3.2b</td>
<td>2.89 ± 0.88b</td>
<td>4.13 ± 0.82c</td>
</tr>
<tr>
<td>S + SA (0.5 mM)</td>
<td>76.46 ± 3.66c</td>
<td>5.76 ± 1.63d</td>
<td>6.76 ± 1.59d</td>
</tr>
<tr>
<td>S + SA (1.0 mM)</td>
<td>70.10 ± 2.91c</td>
<td>4.33 ± 0.74c</td>
<td>5.89 ± 1.13b</td>
</tr>
<tr>
<td>Significance with df 4 and 49</td>
<td>* * *</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

The results are based on 10 replicates for each treatment. Duncan multiple range test was applied to get mean values.

(*) Significant or (NS) non-significant at 0.05% probability level

S= Salt (120 mM NaCl) and SA= salicylic acid

Effect of foliar application of salicylic acid on shoot length, shoot diameter and leaf area under field or pot condition

Shoot length and shoot diameter (cm)

There was gradual increase in shoot length and diameter by increasing salicylic acid concentration. There was maximum increase at concentration of 0.5 mM of SA. Salt stressed plants have shoot length of 42.36cm. It was increased maximum up to 74.35cm at 0.5 mM SA with 120 mM salt treatment. Similarly, shoot diameter was also increased up to maximum at 0.5 mM concentration of SA. Shoot diameter was 2.64, 1.25, 1.65, 2.24 and 2.05cm at control, 120 mM salt, 0.1, 0.5 and 1.0 mM of salicylic acid treatments (Fig. 1a-e).

Leaf area

In control the leaf area is 386 cm² while the leaf area of the plants grown under salt stress was 157 cm². There was a gradual increase in leaf area with gradual increase of salicylic acid concentration. Maximum leaf area was observed when the plants were subjected to concentration of 1.0 mM of SA. Leaf area at 0.1, 0.5, 1.0 mM is 245, 297, 305 cm², respectively.

Table II: Effect of foliar application of SA (Salicylic acid) on shoot length/diameter and leaf area of salt-stressed maize plants.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot length (cm)</th>
<th>Stem diameter (cm)</th>
<th>Leaf area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86.12 ± 5.42e</td>
<td>2.64 ± 0.67e</td>
<td>386 ± 21.63e</td>
</tr>
<tr>
<td>Salt (120 mM)</td>
<td>42.36 ± 2.45a</td>
<td>1.25 ± 0.37a</td>
<td>157 ± 9.77a</td>
</tr>
<tr>
<td>S + SA (0.1 mM)</td>
<td>51.43 ± 3.86b</td>
<td>1.65 ± 0.60b</td>
<td>245 ± 22.43b</td>
</tr>
<tr>
<td>S + SA (0.5 mM)</td>
<td>74.35 ± 2.95d</td>
<td>2.24 ± 0.87d</td>
<td>297 ± 24.65c</td>
</tr>
<tr>
<td>S + SA (1.0 mM)</td>
<td>67.52 ± 3.57c</td>
<td>2.05 ± 0.49c</td>
<td>305 ± 20.21d</td>
</tr>
<tr>
<td>Significance with df 4 and 49</td>
<td>* * *</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

The results are based on 10 replicates for each treatment. Duncan multiple range test was applied to get mean values.

(*) Significant or (NS) non-significant at 0.05% probability level

S= Salt (120 mM NaCl) and SA= salicylic acid

Fig. 1a: Control of maize grown with tap water showing growth without salt stress.
Effect of foliar application of salicylic acid on plant biomass production

Shoot fresh and dry weights

Foliar spray with salicylic acid resulted in an increase in the biomass of maize plants that were grown under saline conditions (Table III). There is gradual increase of biomass with gradual increase of SA concentration. Fresh weight of shoot was maximum (389.24 g) of control and that of under 120 mM salt stress was 211.54g. When SA was applied to salt treated plants it increase the fresh biomass of plant as compared to salt treated ones. However, there was gradual increase in fresh weight with increasing SA level. But it was maximum at 0.5 mM concentration of SA. Shoot fresh weight was 209.23, 341.10, 321.75g at concentration levels 0.1, 0.5 and 1.0 mM of SA, respectively. Same pattern was seen in case shoot dry weight. Shoot dry weight was maximum at 0.5 mM of SA which was 55.88 g. Shoot dry weight of control is 72.56g and that of under salt stress was 30.21g. Then there was gradual enhancement in dry weight of shoot with increase in SA treatment concentrations.

Root fresh and dry weights

A little improvement in was seen fresh and dry weight of root with increasing concentrations of SA under salt stress in maize plants. Root fresh weight was 105.52g of control plant and that of under 120 mM salt stress was 48.88g. There was little increase in fresh weight with increasing concentrations of SA under salt stress. Root fresh weight was maximum at 0.5 mM of SA. Fresh weights were 58.36, 66.51 and 61.60 g at concentrations 0.1, 0.5 and 1.0 mM of SA. In the same pattern, there was a small enhancement in dry weight of root with increasing levels of SA. Dry weight of root of control was 19.25g and that of salt stressed plants was 8.48g. A little increase in dry weight with increase in SA concentrations was noticed. Dry weight of root was maximum at 0.5 mM (12.26g) treatment of SA under saline conditions.
Effect of foliar application of salicylic acid on soluble protein contents

Table III: Effect of foliar spray of Salicylic Acid on biomass production of salt-stressed maize plants

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
<th>Root weight (g)</th>
<th>Fresh wt. (g)</th>
<th>Dry wt. (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>389.24 ± 15.12</td>
<td>72.56 ± 3.44</td>
<td>108.52 ± 4.27</td>
<td>19.25 ± 1.28</td>
<td></td>
</tr>
<tr>
<td>Salt (120 mM)</td>
<td>211.54 ± 8.73</td>
<td>30.21 ± 1.23</td>
<td>48.88 ± 3.85</td>
<td>8.48 ± 1.67</td>
<td></td>
</tr>
<tr>
<td>S+ SA (0.1 mM)</td>
<td>290.23 ± 7.14</td>
<td>38.33 ± 2.40</td>
<td>58.36 ± 2.33</td>
<td>10.34 ± 1.16</td>
<td></td>
</tr>
<tr>
<td>S+ SA (0.5 mM)</td>
<td>341.10 ± 10.45</td>
<td>55.88 ± 4.38</td>
<td>66.51 ± 3.41</td>
<td>12.26 ± 1.77</td>
<td></td>
</tr>
<tr>
<td>S+ SA (1.0 mM)</td>
<td>321.75 ± 6.47</td>
<td>52.43 ± 2.45</td>
<td>61.60 ± 2.28</td>
<td>11.22 ± 1.14</td>
<td></td>
</tr>
<tr>
<td>Significance with df 4 and 49</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

The results are based on 10 replicates for each treatment.

Duncan multiple range test was applied to get mean values.

(*) Significant or (NS) non-significant at 0.05% probability level.

Effect of foliar application of salicylic acid on peroxidase activity

There is clear impact of SA on peroxidase activity of plants under saline conditions. In control plants peroxidase activity was 0.16 mg/g tissue and in salt stressed plants was 0.13 mg/g tissue. This result shows that there is decrease in activity under salt stress. But with treatments of SA there is positive effect on peroxidase activity. Its value is 0.16, 0.15 and 0.15 mg/g tissue with concentration levels (0.1, 0.5 and 1.0 mM) of salicylic acid, respectively (Table IV).

Effect of foliar application of salicylic acid on proline contents

There was significant impact of salts on proline contents of maize plants. In control (plants) its value was 16.19 µmol/g of FW but under salt stress its value increased up to 38.26 µmol/g of FW. These contents were decreased at 0.1 mM concentration of SA but at higher concentrations of SA proline contents were again increased. Proline contents at 0.1, 0.5 and 1.0 mM concentrations of SA were 30.53, 47.44 and 58.69 µmol/g of FW, respectively (Table IV).

Effect of foliar application of salicylic acid on activity of superoxide dismutase

Data shows that superoxide dismutase (SOD) activity of maize plants increased significantly under salt stress. In control plants it was 55.43 U/mg of protein and in salt stressed plant it was 81.24 U/mg of protein. But there was little decrease in SOD activity with increasing SA level in salt stressed plants. SOD activity was 72.36, 69.44 and 66.5 U/mg of protein noticed at 0.1, 0.5 and 1.0 mM concentrations of SA, respectively.

Table IV: Effect of foliar spray of SA (Salicylic acid) on soluble protein contents, peroxidase, superoxide dismutase activities and proline contents of salt-stressed maize plants

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Soluble Protein Contents (mg/g tissue)</th>
<th>Peroxidase activity (mg/g tissue)</th>
<th>Superoxide dismutase activity (U/mg protein)</th>
<th>Proline Contents (µmol/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.23 ± 0.006a</td>
<td>0.16 ± 0.007c</td>
<td>55.43 ± 12.12a</td>
<td>16.19 ± 0.742c</td>
</tr>
<tr>
<td>Salt (120 mM)</td>
<td>0.34 ± 0.014d</td>
<td>0.13 ± 0.005a</td>
<td>81.24 ± 8.68e</td>
<td>38.26 ± 3.24c</td>
</tr>
<tr>
<td>S+ SA (0.1 mM)</td>
<td>0.32 ± 0.009b</td>
<td>0.16 ± 0.004c</td>
<td>72.36 ± 11.43d</td>
<td>30.53 ± 5.66b</td>
</tr>
<tr>
<td>S+ SA (0.5 mM)</td>
<td>0.28 ± 0.004c</td>
<td>0.15 ± 0.005c</td>
<td>69.44 ± 4.27c</td>
<td>47.44 ± 6.22c</td>
</tr>
<tr>
<td>S+ SA (1.0 mM)</td>
<td>0.36 ± 0.006b</td>
<td>0.15 ± 0.006b</td>
<td>66.57 ± 5.09b</td>
<td>58.69 ± 5.46b</td>
</tr>
<tr>
<td>Significance with df 4 and 49</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
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