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Research Article

# Knowledge and attitude towards antibiotic use and awareness on antibiotic resistance among older people in Malaysia

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**Abstract:** Antibiotic resistance is a significant global health concern. The challenges of antibiotics usage among elderly include antibiotic overuse, antibiotic resistance, dosing of antibiotics and adverse effects. This study aimed to evaluate the knowledge and attitude towards antibiotics usage among the elderly as well as the awareness on antibiotic resistance. A prospective cross-sectional study was conducted in outpatient department of a tertiary hospital in Malaysia from September 2016 to November 2016 using researcher-assisted and validated questionnaires. A total number of 250 elderly patients were recruited and majority of them (n=152, 60.8%) had moderate level of knowledge, with a median (IQR) score of 6.00(3.00) out of 12 points and positive attitude with a median (IQR) score of 7.00(1.00) out of 8 points. There was a positive correlation between knowledge and attitude scores ($r = 0.335$, $p<0.001$). Generally, awareness on antibiotic resistance was low as majority of the elderly respondents were not familiar (n=182, 71.8%) with the term "antibiotic resistance" and had never hear or read about antibiotic resistance before (n=150, 60.0%). In conclusion, majority of the elderly respondents had moderate level of knowledge and positive attitude towards antibiotics, implying the necessities of having public awareness campaigns to rectify the incomplete understanding of antibiotic use.

**Keywords:** Antibiotic resistance, Antibiotic use, Elderly

## 1. INTRODUCTION

Antibiotics were one of the largest groups of drugs being utilized in the health care system other than anti-diabetic, anti-hypertensive and lipid-lowering agents. There were temporal relationship between the increased use of antibiotics over the years and the growing rates of antimicrobial resistance worldwide. Inappropriate antimicrobial use has been associated with increased morbidity, mortality and hospital costs [1]. There were several issues related to proper antibiotic use, which potentially affecting the delivery of quality and effective healthcare [2]. Patients are the end consumers of all medical treatments, including antibiotics [3]. However, misconceptions about antibiotics existed. People deem antibiotics as powerful medicines and always capable of preventing and treating most of the perceived illness [4].

The irrational use of antibiotics will result in the emergence of resistant bacterial strains that adding the burden on national health system. Older people were the most vulnerable population as they knew less about medications as compared to the young [5]. The older adults are more susceptible to certain infections as compared to the young; hence they tend to have more serious infections with atypical and non-specific clinical manifestations [6]. The challenges in the use of antibiotics in older people include antibiotic overuse, antibiotic resistance, dosing of antibiotics and adverse effects. Antibiotic resistance also is associated with a high mortality risk and increased economic costs. As a result of population ageing, there is an urge to address issues of older adults so that more information on this population will be available [7].

To date, there is a lack of information regarding antibiotic usage among the older people, along with their knowledge about, attitude toward and awareness on antibiotics use and resistance. Therefore, this

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study aimed to evaluate the knowledge and attitude towards antibiotic use among older people. This study also aimed to investigate the awareness of older people on antibiotic resistance and their perceptions about patient-doctor relationship on antibiotic prescribing.

## 2. MATERIALS AND METHODS

### 2.1 Study design

A cross-sectional study was conducted among older people from out-patient pharmacy departments in a tertiary hospital in Malaysia from September to November 2016 using researcher-assisted and validated questionnaire upon their informed consent. Respondents were recruited based on the following criteria; aged 60 years old and above who are seeking treatment in that medical center and aware of the term "antibiotic". Those who did not complete the questionnaire were excluded from the study. This study was approved by our Institutional Human Research Ethics Committee.

A set of validated instruments was utilized in this study. The first section of this questionnaire was to document respondents' recent antibiotic usage within the past three months [8]. An arbitrary scoring system was employed according to the answer provided. The knowledge score was calculated as a continuous variable by summing the respondents' number of correct answers to 12 statements. The original Bloom's cut-off points, 80.0%–100.0%, 60.0%–79.0%, and ≤59.0%, were adapted and modified from another study [9]. The scores for knowledge varied from 1 to 12 points and were classified into three levels as follows: 1. high level: 9–12 scores; 2. moderate level: 5–8 scores; and 3. low level: 0–4 scores. Another section of this questionnaire consisted of 8 statements to assess respondents' attitudes towards antibiotic usage. The attitude statements were used to address usage of antibiotics during common colds, patients' expectation of their doctor when they are suffering from common colds, completion of antibiotic treatment course, sharing of antibiotics with family members, keeping antibiotics stocks at home for emergency use, usage of leftover antibiotics, compliance with the instructions on the label in taking antibiotics and the awareness of expiry date before taking the antibiotics. Respondents were required to answer according to a five point Likert scale ranging from "Strongly agree" to "Strongly disagree". Those who answered "Strongly agree" and "Agree" were classified as "agreed" and those who answered "Strongly disagree" and "Disagree" as having disagreed in order to simplify the results' presentation and analysis. The attitude score was also calculated as a continuous variable by summing the respondents' number of positive responses to eight statements. One point will be given for each positive response, whereas zero point will be given for each negative or uncertain response. Total attitude scores ranged from zero to eight, with a higher total score indicating a more positive attitude towards antibiotic usage.

Eight questions were also included to assess the awareness of respondents on antibiotic resistance [10]. Respondents were required to answer "Yes", "No" or "Not sure" to the statements related to antibiotic resistance. For those who had heard or read about antibiotic resistance, they were required to provide further information regarding the source of information about antibiotic resistance. The last section included six statements to explore respondents' perception about patient-doctor relationship in antibiotic prescribing [11]. Responses were measured using a five point Likert scale ranging from "Strongly disagree" to "Strongly agree". To simplify the results' presentation and analysis, those who answered "Strongly agree" and "Agree" were classified as "agreed" and those who answered "Strongly disagree" and "Disagree" as having disagreed.

All data analyses were performed using Statistical Package for Social Science (SPSS) version 22.0. Demographic characteristics, recent usage of antibiotics, knowledge of and attitude towards antibiotic, awareness on antibiotic resistance and perception about patient-doctor relationship on antibiotic prescribing were summarized using descriptive statistics. Shapiro-Wilk's test was used to determine the normality of knowledge and attitude scores. Chi-square test was used to determine association between respondents' demographic characteristics and level of knowledge regarding antibiotics. In addition, Mann-whitney U Test and Kruskal-Wallis H Test were performed to determine the differences in attitude scores among the respondents with different demographic

characteristics, where appropriate. Post hoc test was done after Kruskal-Wallis H Test for pairwise comparison of attitude scores among respondents with different demographic characteristics. Besides, Spearman's rank correlation analysis was performed to examine the degree of relationship between knowledge and attitude score. Chi-square test was used to determine association between level of knowledge and awareness on antibiotic resistance. Correlation between related statements was performed using Chi-square test. In all statistical analyses, a p-value of less than 0.05 was considered to be statistically significant.

## 3. RESULTS AND DISCUSSION

A total of 250 subjects were included in the study (**Table 1**). The median (interquartile, IQR) age was 69(8) years, with most falling within 65-69 age group (n= 87, 34.8%). A total of 55 respondents (22.0%) reported using antibiotics within the past three months of the survey; most of whom obtained their antibiotics as prescribed and given by clinic or hospital after consultation with doctors (98.2%). In this study, the knowledge score about antibiotics use ranged from 1 to 12 points, with a median (IQR) score of 6.00(3.00). Majority of the respondents (n=152, 60.8%) had moderate level of knowledge. Overall, the level of education ($p<0.001$) and total number of medication taken ($p=0.015$) were found to have significantly positive associations with the level of knowledge towards antibiotics.

More than half of the respondents did not know that antibiotics are not effective in treating viral infection or common cold and cough (**Table 2**). For the knowledge regarding identification of antibiotic, most of the respondents correctly answered that antibiotics are not the same as other medications that are used to relieve pain and fever such as

**Table 1.** Association of respondents' demographic characteristics with level of knowledge to the antibiotic use (N=250)

| Demographic characteristics | | Frequency, n (%) | Level of knowledge | | | |
|---|---|---|---|---|---|---|
| | | | Poor n (%) | Moderate n (%) | Good, n (%) | p-value |
| **Age (in years)** | 60-64 | 55 (22.0) | 7(12.7) | 34(61.8) | 14(25.5) | 0.251[a] |
| | 65-69 | 87 (34.8) | 21(24.1) | 54(62.1) | 12(13.8) | |
| | 70-74 | 59 (23.6) | 9(15.3) | 37(62.7) | 13(22.0) | |
| | 75 or older | 49 (19.6) | 14(28.6) | 27(55.1) | 8(16.3) | |
| **Gender** | Male | 162 (64.8) | 34 (21.0) | 96(59.3) | 32(19.8) | 0.787[a] |
| | Female | 88 (35.2) | 17 (19.3) | 56(63.6) | 15(17.0) | |
| **Educational level** | Primary school | 59 (23.6) | 22 (37.3) | 31(52.5) | 6 (10.2) | 0.001 [b**] |
| | Secondary school | 118 (47.2) | 25 (21.2) | 69(58.5) | 24(20.3) | |
| | College/ University | 66 (26.4) | 2 (3.0) | 47(71.2) | 17(25.8) | |
| | None | 7 (2.8) | 2 (28.6) | 5 (71.4) | 0 (0.0) | |
| **Common healthcare location** | Government clinic/hospital | 238 (95.2) | 48 (20.2) | 147(61.8) | 43(18.1) | 0.836 [b] |
| | Private clinic/ hospital | 10 (4.0) | 3 (30.0) | 4 (40.0) | 3 (30.0) | |
| | Others | 2 (0.8) | 0 (0.0) | 1 (50.0) | 1(50.0) | |
| **Total medication taken** | 1-4 | 109 (47.6) | 14 (12.8) | 69 (63.3) | 26(23.9) | 0.015[a*] |
| | 5 and more | 120 (52.4) | 32 (26.7) | 71 (59.2) | 17(14.2) | |

a=chi square test, b=Yate's correction of chi square, ** Significant at the 0.01 level (2-tailed), *Significant at the 0.05 level (2-tailed)

**Table 2.** Percentage of answers based on knowledge statements regarding antibiotics (N=250)

| Statement | Correct answer, n (%) | Incorrect answer, n (%) | Unsure, n (%) |
|---|---|---|---|
| **Role of Antibiotic** | | | |
| 1. Antibiotic are medicine that can kill bacteria. | 215 (86) | 11 (4.4) | 24 (9.6) |
| 2. Antibiotic can be used to treat viral infection. | 41 (16.4) | 144 (57.6) | 65(26.0) |
| 3. Antibiotic work on most cold and cough. | 76 (30.4) | 143 (57.2) | 31(12.4) |
| **Identification of antibiotic** | | | |
| 4. Penicillin is an antibiotic. | 98 (39.2) | 51 (20.4) | 101(40.4) |
| 5. Antibiotic are the same as medications used to relieve pain and fever such as aspirin and paracetamol. | 166 (66.4) | 42 (16.8) | 42 (16.8) |
| **Good Bacteria** | | | |
| 6. Antibiotic can kill bacteria that normally live on skin and gut. | 140 (56.0) | 20 (8.0) | |
| 7. Bacteria that normally live on the skin and in the gut are good for your health. | 72 (28.8) | 88 (35.2) | |
| **Adverse effect** | | | |
| 8. Antibiotic may cause allergic reactions | 192 (76.8) | 23 (9.2) | 35 (14.0) |
| 9. Antibiotic does not cause side effects | 165 (66.0) | 44 (17.6) | 41 (16.4) |
| 10. Overuse of antibiotics can cause antibiotic to lose effectiveness in long term. | 204 (81.6) | 7 (2.8) | 39 (15.6) |
| **Administration of antibiotic** | | | |
| 11. It is okay to stop taking antibiotic when symptoms are improving. | 117 (46.8) | 129 (51.6) | 4(1.6) |
| 12. Taking less antibiotics than prescribed is more healthy than taking the full course prescribed. | 165 (66.0) | 55 (22.0) | 30(12.0) |

aspirin and paracetamol. More than half of the respondents knew that antibiotic can kill bacteria that normally live on skin and gut, however, most of them did not agree that or were unsure whether these bacteria are good for their health. In terms of adverse effect of antibiotic, more than half of them respondents were aware that antibiotics may cause allergic reactions or side-effects. About all of the participants correctly answered that antibiotic resistance may occur due to overuse of antibiotics. Nevertheless, more than half of the respondents thought it is fine to stop taking antibiotic when symptoms are improving.

The attitude score towards antibiotics use ranged from 1 to 8 points, with a median (IQR) score of 7.00(1.00) (**Table 3)**. Overall, gender (p=0.022), level of education (p=0.002) and total number of medication taken (p=0.015) were found to contribute significantly to the attitude score. Mann-Whitney U Test reported that the attitude score of female respondents (mean rank = 139.36) was significantly higher (p=0.022) than the male respondents (mean rank = 117.97). Kruskal-Wallis H Test reported a significant difference (p=0.002) in attitude score among respondents with different level of education. Post hoc analysis revealed that the attitude score of respondents with primary education was significantly lower (p<0.001) than the attitude score of respondents with secondary education and tertiary education. Meanwhile, Mann-Whitney U Test reported that the attitude score of older people who taking 1 to 4 medications (mean rank = 125.86) was significantly higher (p=0.015) than the attitude score of the respondents taking 5 and more medications (mean rank = 105.13).

In general, our respondents were found to have positive attitudes towards antibiotics with results showing positive responses of more than half from

**Table 3.** Difference in attitude score in respondents with different demographic characteristics (N=250)

| Demographic characteristics | | Attitude score | |
|---|---|---|---|
| | | Mean rank | p-value |
| **Age (in years)** | 60-64 | 133.75 | 0.410§ |
| | 65-69 | 130.80 | |
| | 70-74 | 115.40 | |
| | 75 or older | 118.99 | |
| **Gender** | Male | 117.97 | 0.022‡* |
| | Female | 139.36 | |
| **Employment status** | Employed | 87.00 | 0.116§ |
| | Housewife/househusband | 138.79 | |
| | Retired/unemployed | 123.64 | |
| **Common healthcare location** | Government clinic/hospital | 125.95 | 0.776§ |
| | Private clinic/hospital | 121.60 | |
| | Others | 91.50 | |
| **Total medication taken** | 1-4 | 125.86 | 0.015‡** |
| | 5 and more | 105.13 | |

** Significant at the 0.01 level (2-tailed)
* Significant at the 0.05 level (2-tailed)
‡ Mann-Whitney U Test
§ Kruskal-Wallis H Test
Post hoc test for pairwise comparison of groups-"ab" and "ac" showed statistically significant difference with $p < 0.001$.

all attitude statements (**Table 4).** Nevertheless, one fifth of the respondents will take antibiotics when they get cold in order to recover from illness. Besides, a portion of the respondents expected antibiotic to be prescribed by their doctors if they suffer from common cold symptoms. More than

**Table 4.** Attitude statements regarding antibiotics usage (N=250)

| Statement | Agree, n (%) | Neutral, n (%) | Disagree, n (%) | Median (IQR) |
|---|---|---|---|---|
| 1. When I get cold, I will take antibiotics to help me get better more quickly. | 52 (20.8) | 24 (9.6) | 174 (69.6) | 3(1) |
| 2. I expect antibiotic to be prescribed by my doctor if I suffer from common cold symptoms. | 72(28.8) | 26 (10.4) | 152 (60.8) | 3(2) |
| 3. I normally stop taking an antibiotic when I start feeling better. | 94 (37.6) | 11 (4.4) | 145 (58.0) | 3(2) |
| 4. If my family member is sick I usually will give my antibiotic to them. | 5 (2.0) | 4(1.6) | 241 (96.4) | 3(0) |
| 5. I normally keep antibiotic stock at home in case of emergency. | 14 (5.6) | 5(2.0) | 231 (92.4) | 3(0) |
| 6. I will use leftover antibiotics for a respiratory illness (runny nose/ sore throat / flu). | 29(11.6) | 16 (6.4) | 205 (82.0) | 3(0) |
| 7. I will take antibiotic according to the instruction on the label. | 241 (96.4) | 8 (3.2) | 1 (0.4) | 1(0) |
| 8. I normally will look at the expiry date of antibiotic before taking it. | 208 (83.2) | 18 (7.2) | 24(9.6) | 1(0) |

**Table 5.** Awareness on antibiotic resistance (N=250)

| | Yes, n (%) | No, n (%) | Unsure, n(%) |
|---|---|---|---|
| 1. Are you familiar with the term 'Antibiotic Resistance'? | 54 (21.6%) | 182 (71.8%) | 14 (5.6%) |
| 2. Have you ever heard or read about Antibiotic Resistance? | 81 (32.4%) | 150 (60.0%) | 19 (7.6%) |
| 3. Do you think this is an important healthcare issue? | 188 (75.2%) | 6 (2.4%) | 56 (22.4%) |
| 4. Has your doctor ever talked to you about Antibiotic Resistance? | 56 (22.4%) | 187 (74.8%) | 7 (2.8%) |
| 5. Has your doctor ever given your reading materials about Antibiotic Resistance? | 9 (3.6%) | 237 (94.8%) | 4 (1.6%) |
| 6. Does your pharmacist talk to you about your prescription medications? | 210 (84.0%) | 28 (11.2%) | 12 (4.8%) |

one third of the respondents admitted to stop taking an antibiotic when they began to feel better from their condition. There was a significant correlation between knowledge score and attitude score ($r = 0.335$, $p<0.001$) implying that a higher knowledge score was associated with a more positive attitude score. The knowledge statement "Antibiotics work on most colds and coughs" was significantly associated with the attitude statement "When I get cold, I will take antibiotics to help me get better more quickly" ($p<0.001$) and "I expect antibiotic to be prescribed by my doctor if I suffer from common cold symptoms" ($p=0.019$).

Nevertheless, only less than quarter of the respondents were familiar with the term "antibiotic resistance" (**Table 5**). A majority of respondents were of the opinion that doctors should inform them about antibiotic resistance. Of those respondents who had heard or read about antibiotic resistance, most of them reported that they obtained this information through media, followed by information from doctors. A significant association was found between the level of knowledge and awareness on antibiotic. Older people who were familiar with the term "antibiotic resistance" ($p<0.001$), ever heard or read about antibiotic resistance ($p<0.001$) and felt antibiotic resistance as an important healthcare issue ($p<0.001$) had significantly higher level of knowledge towards antibiotics. Most of the respondents agreed that pharmacists often tell them how antibiotics should be used and doctors often spend a good time to inform them how antibiotics should be used during the consultation. Majority of respondents trusted the doctors' decision whether or not they were being prescribed with antibiotics. More than half of the respondents did not agree that doctors will be affected by patients' expectation in antibiotic prescribing. Lastly, less than half of the older people agreed that doctors often time to consider carefully whether antibiotics are needed or not (**Table 6**).

**Table 6.** Perception about patient-doctor relationship on antibiotic prescribing (N=250)

| Statement | Disagree, n (%) | Unsure, n (%) | Agree, n (%) | Median (IQR) |
|---|---|---|---|---|
| 1. Pharmacists often tell you how antibiotics should be used. | 62 (24.8) | 28 (11.2) | 160 (64.0 ) | 3(1) |
| 2. Doctors often take time to inform you during the consultation how antibiotics should be used. | 32 (13.2) | 25 (10.0) | 192 (76.8) | 3(0) |
| 3. I trust the doctor decision if she or he decides not to prescribe antibiotic. | 3 (1.2) | 18 (7.2) | 229 (91.6) | 3(0) |
| 4. I trust the doctor's decision when she or he prescribes antibiotics. | 4 (1.6) | 20 (8.0) | 226 (90.4) | 3(0) |
| 5. Doctors often prescribe antibiotics because the patient expects it. | 164 (65.6) | 43 (17.2) | 43 (17.2) | 1(1) |
| 6. Doctors often take time to consider carefully whether antibiotics are needed or not. | 54 (21.6) | 75 (30.0) | 121 (48.4) | 2(1) |

In our study, low level of knowledge about antibiotics was found among older people with primary school education, suggesting patients with higher educational level had higher accessibility to health information through advanced sources including books, electronic article, social media or direct communication with their health care professionals [12]. Multiple medications can result in increased complexity of medication regimen, leading to the difficulties in remembering the name and purposes of medications. Misconceptions about the role of antibiotics in treating viral infection and most of the cough and cold were prevalent among the older people. Such misconceptions could be attributed to the fact that the general terms such as "germ" or "microbes", instead of "bacterial" and "virus" were commonly used by healthcare providers in providing medical advice to the patients without medical background [13]. Proper patient education was then suggested to correct the misconceptions about the role of antibiotics among the older people.

Most of older people were not familiar with the identity of penicillin as an antibiotic. This was not surprising as older people relied mostly on pill size, shape and color to recognize the medications instead of the actual names [14]. Nevertheless, most of the respondents were able to differentiate antibiotics from analgesics and antipyretics such as aspirin and paracetamol. It was possible that they used to differentiate antibiotics from other classes of drugs according to the perceived efficacy of antibiotics, but not the pharmacological action of antibiotics. It was observed that older people might not really understand the importance of completing full course of antibiotic regimen. Poor compliance to antibiotic regimen may result in undesirable treatment outcomes as patients may experience recurrent infection, which further worsening their health conditions. Hence, healthcare professionals should take sufficient steps in tackling antibiotic compliance issues among the older people by providing adequate and relevant patient education.

Female were found to have a more positive attitude towards antibiotic usage than the male older people. Some studies showed that older people women utilized more healthcare services than older people men; it may indirectly reflect their greater concerns in health-related problems and better attitude towards medication usage [15-16]. With higher level of education, patients were able to understand more about their health needs as well as comply with the medical instructions provided by healthcare professionals [17]. Older people who took higher number of medications were also found to have more negative attitude towards antibiotic usage. This can be explained by the complexity of medication regimen arised as a result of multiple medications [18-19].

Overall, majority of the older people had a positive attitude towards antibiotic use, Yet, antibiotics were assume to be able to provide a better recovery period for the common cold and older people did expect antibiotics to be prescribed by doctors for their common cold symptoms. The belief on "antibiotics work on most cough and cold" implied that the expectations of antibiotics during common colds were built upon the perceived effectiveness of antibiotics in relieving coughs and colds. Patients' expectation of antibiotics can be also affected by their past experience. For example, patients who have taken antibiotics for their common colds before this will have misperceptions that antibiotics were effective, although common colds were generally self-limiting [20]. Alternatively, some patients may expect an antibiotic to be prescribed by doctors to compensate for the time and effort spent to visit the doctor and to avoid multiple doctor visit when their illness deteriorated [21].

Prematurely stopping the antibiotic treatment was a common mistake among patients when they have experienced symptomatic relief, especially when the infections are mild in nature [22]. Nevertheless, it was encouraging to observe that majority of the older people showed high disagreement to the wrong practice of using antibiotics such as sharing of antibiotics with family members or using leftover antibiotics. Also, it was found out that the older people were less able to understand the medical instructions on the medication labels as compared to the young [23]. Thus, consistent, complete, organized and standardized information on medication labels are important to improve readability and understanding of patients on the administration of medication safely.

Only a quarter of our respondents was familiar with the term "Antibiotic Resistance" or came across with the term prior to this study. In western countries, patients were more exposed to the issues and they also perceived antibiotic resistance as an important healthcare issue [9]. Generally, older people who were more aware of antibiotic resistance had a higher level of knowledge about antibiotics. Inadequate awareness on antibiotic resistance can stimulate more inappropriate antibiotic use and further accelerate the antibiotic resistance [3]. It was notable that discrepancy existed between patients' expectations and the actual information about antibiotic resistance provided by their healthcare professionals. Many believed that their doctors should be the one that inform them about antibiotic resistance, however few received such information. Most people relied on media to provide information about this issue. These findings addressed the importance of involvement of healthcare professionals in informing patients regarding causes and consequences of antibiotic resistance. Educating the older people regarding appropriate antibiotic use can lead to better health outcomes and slow down antibiotic resistance [9].

Good relationship between patients and attending doctors are important as older people would able to voice their concerns on antibiotics directly. This was encouraging as trust is one of the main components in maintaining patient-doctor relationship [24]. Furthermore, majority of our older people also disagreed that doctors often prescribe antibiotics upon patients' expectation although almost one third of them expected antibiotics to be prescribed by doctors for common cold symptoms.

## 4. CONCLUSIONS

This study was to assess the knowledge and attitude towards antibiotics use and resistance among older people, highlighting the necessities of having public awareness campaigns to rectify the incomplete understanding of antibiotic uses. Nevertheless, our findings were limited as those who did not aware of the term "antibiotic" were excluded in the beginning of the recruitment process, giving potential risk of missing of important information about older people in relation to antibiotic uses. Our results showed that majority of the older people had moderate level of knowledge about and positive attitude towards antibiotics. Misunderstandings regarding the effectiveness of antibiotic in treating viral infection and common cold symptoms existed. Majority of the older people were not aware of the antibiotic resistance, but they perceived it as an important healthcare issue. In addition, good patient-doctor relationship was observed in this study. Further study can be conducted to investigate the impact of antibiotic awareness campaign and patient counseling on rational antibiotic usage among the older people, such as misconceptions about antibiotics, compliance to antibiotic regimen and unnecessary expectation of antibiotics.

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Research Article

# Formulation, Characterization and *In-vitro* Sun Protection Factor of a Lemongrass Sunscreen Lotion

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**Abstract:** Sunburn, pigmentation disorders, photocarcinogenesis, immunosuppression and other skin related damages are caused by the ultraviolet radiation present in the sunlight. The aim of this study was to determine the sun protection factor (SPF) value of the lemongrass extract, also the formation of its stable sunscreen lotion and to determine its SPF value. For the determination of SPF value of the developed formulation, spectrophotometric method was used along with Mansur equation. The SPF value of lemongrass sunscreen lotion was 22 with the antioxidant potential of 94.20%. The hydrogen ion concentration (pH) of the lotion was 5.5, which complied with skin pH. Viscosity profile of lotion indicated good rheology which is an important aspect of a formulation during application. The formulation was stable as there was no phase separation observed after centrifugation, freeze-thaw and thermal stress tests. Harmful effects of chemicals are evident and therefore, the natural source could become a good, economical, easily accessible and safe alternative formulation ingredient in sunscreen products due to its beneficial effects and safety.

**Keywords:** Emulsions, formulation/stability, lemongrass, spectroscopy, sun protection, sunscreen

## 1. INTRODUCTION

Skin, the outermost layer of the body that acts as a sensory organ is a part of the integumentary system that defends the body from surrounding environment. Epidermis, dermis and hypodermis are the three main layers of the skin [1]. Melanin naturally present in the skin is responsible for protection of skin from the harmful effects of sun [2]. Skin aging is not hazardous for a person but it can have negative effects on psychology of a person. The interaction of the skin with environment directly or indirectly is the main cause of premature ageing [3]. Photo carcinogenesis, inflammation, erythema, immunosuppression, pigmentation, photo aging, hyperplasia and vitamin D synthesis are certain skin responses induced by ultraviolet radiations (UV-R). [4]. The radiations from sun comprises of 50% visible light lying in the range of 400-800 nm, 40% infrared radiation lying in the range of 1300-1700 nm and 10% UV-R lying in the range of 100-400 nm [5-6].

The substances that absorb or block UV rays of sunlight are called sunscreens or sun blocks. All compounds used as sunscreen filter are by their nature and chemicals that can absorb UV-A and / or UV-B light [7]. The ideal sunscreen product should provide good protection throughout the whole range of UV spectrum, even after sunlight exposure. An ideal sunblock should be non-irritating, non-toxic and not produce any type of allergy [4]. Due to antioxidant power and UV-R absorption, natural ingredients extracted from plants have been recently considered as potential sunscreen resources [8].

The measurement of the effectiveness of sunblock in protecting the human skin from UV-R is called Sun protection factor (SPF). **Table 1** shows various ratings of SPF. To prevent any possible

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**Table 1.** Sun Protection Factor Ratings [9]

| SPF Range | Effective UV- R transmission (%) | SPF Rating | Protection Category |
|---|---|---|---|
| 15-24 | 6.7-4.2 | 15, 20 | Good |
| 25-39 | 4.1-2.6 | 25, 30, 35 | Very Good |
| 40-50, 50+ | Less than 2.5 | 40, 45, 50, 50+ | Excellent |

damage to skin by UV rays, the sunscreen products should have adequate SPF values and a wide range of absorbance between 290 to 400 nm to absorb and reflect enough amount of UV ray photons [10].

Nanotechnology reveals its great potential in the field of research and development by increasing the efficacy of the product. . To overcome certain disadvantages associated with the traditional products, application of nanotechnology is rising in the area of cosmeceuticals [11]. Because natural compounds are capable of attenuating some of the UV-induced aging effects in the skin, increased attention has been generated in the area of cosmetic sciences [12].

The substances which delay or prevent the oxidation of an oxidizable substrate are called the "Antioxidants" They may be synthetic or natural antioxidants. Biological systems produce the natural antioxidants. Natural antioxidants are present in many plants including lemongrass, garlic, turmeric, onions, celery seed, basil, rosemary, ginseng, and coriander [13]. In previous studies, it was concluded that lemon grass has high antioxidant capacity than many botanicals [14] [15].

Principal compounds were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) and identified as neral, nerolic acid, geraniol, geranic acid and geranial in lemongrass oil [16]. Along with other biological activities reported in the literature, potent tyrosinase inhibitory activity has been found in lemongrass. Geranic acid was discovered as one of the active substances through assay-guided purification [17] and percentage values of geranic acid have been found to be 0.3%, 1.1%, 0.4% in various studies[18] [19]. As lemongrass has antioxidant potential, the objective of this study was to determine the SPF value of lemongrass extract as well as to make the sunscreen lotion of lemongrass and to check its SPF value.

## 2. MATERIALS AND METHODS

### 2.1 Materials

All chemicals used were of analytical grade. Materials used in the study were Lemongrass (Local market, Lahore, Pakistan), Ethanol (Riedel-de Haen, Germany), Salicylic acid (Local market, Lahore, Pakistan), DPPH(2,2-Diphenyl-2picrylhydrazyl) (Sigma), GMS (glyceryl mono stearate) (Local market, Lahore, Pakistan), Cetyl alcohol (BDH, U.K), Silicone oil (Local market, Lahore, Pakistan), Glycerin (Riedel-de Haen, Germany), Bees wax (Local market, Lahore, Pakistan), Ascorbic acid (Sigma, Germany), Potassium hydroxide (Omicron science ltd, U.K), Tween 80 (Merck-Schuchardt, America), Palmitic acid (Sigma-Aldrich, Germany), Stearic acid (Daejung, Korea), Amaranth red (U.S.A) and Distilled water (Research lab University of Central Punjab, Lahore, Pakistan).

### 2.2 Plant collection and identification

Mature, healthy and disease-free lemongrasses (*Cymbopogon citratus*) plant grown in a nursery of Lahore were collected and shade-dried leaves were used in the present study. For the identification of the lemongrass, the plant was provided to a botanist for confirmation of its taxonomical classification. Voucher specimens (voucher no. GC. Herb. Bot.3319) were deposited in the herbarium maintained by Government College University Lahore.

### 2.3 Preparation of lemon grass extract

100 gm of lemon grass was weighed precisely on the weighing balance (Shimadzu, Japan) and then grinded into blender into coarse powder, which was then transferred to an amber, colored bottle having a capacity of 2.5 L. For the extraction purpose, ethanol and water were used as menstruum in a ratio

of 70:30. For the menstruum preparation, 1400 ml ethanol was transferred to an amber colored bottle after being measured in a measuring cylinder and then 600 ml of distilled water was transferred to the same bottle using a measuring cylinder. Finally, the lemon grass was macerated in 2 L of menstruum with shaking of the bottle on alternate days for a period of 15 days.

**2.4 Filtration and evaporation of extract**

For the filtration of the lemongrass extract, Whatman (grade 54) filter paper was used. The volume obtained after filtration was then concentrated in a rotary evaporator (Heidolph- VAP, Germany). The crude extract was kept in air-tight amber bottle and stored at cold temperature until it was further used [20].

**2.5 Dilution of extracts for SPF determination**

For determination of SPF value, dilution was done, so that the final concentration obtained should be 10,000µg/ml.

**2.6 Determination of SPF value of lemongrass extract**

For the determination of SPF value, absorption spectra of the lemongrass extract was obtained in the range of 290nm to 320nm using the quartz cell. Ethanol was used as blank.

Absorption values for the lemongrass extract were recorded at the wavelength ranging from 290nm to 320nm with an interval of 5nm. Triplicate readings were taken for each wavelength and then their average was calculated which was then used for the determination of SPF by using the Mansur equation [10].

$SPF = CF \times {}_{290}\Sigma^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$

Where EE - erythemal effect spectrum; I-solar intensity spectrum; Abs-Absorbance of sunscreen product; CF-correction factor (=10). The value of EE x I are constant and preset as shown in **Table 1.**

**2.7 DPPH radical scavenging capacity assay**

2, 2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical was used to determine the antioxidant activity as described previously with slight modification [21]. The concentration of DPPH 100 µM in methanol was used. Total assay volume was 100ul, containing 10 µl of the test solution and 90 µl of DPPH solution in a 96 well plate. The contents were mixed and incubated at 37°C for 30 minutes. Synergy HT BioTek® USA microplate reader was used to determine the diminution in absorbance at 517nm. Standard antioxidant used was ascorbic acid (100 – 1000 µg / mL). All experiments were carried out in triplicate.

The percent inhibition was determined by the following formula.

Inhibition (%) = (Abs. of control – Abs. of test solution) / Abs. of control× 100

Where,

Absorbance of Control = Total radical activity without inhibitor.

Absorbance of Extract = Total radical activity in the presence of test/standard compound [21].

**2.8 Preparation of lemongrass lotion**

13 trials were done to get the desired formulation as shown in **Table 2**. Different concentrations of the oil phase and aqueous phase were prepared to get the right consistency of the formulation. A selection criterion was designed to select the optimal dosage form. Method adopted for the formation of the desired formulation is described below. Oil phase i.e. glyceryl mono- stearate (GMS) and silicone oil were transferred to a beaker which was then placed in water-bath (Jiangsu Zhengji instruments CO. LTD, China) having a temperature of 75ºC for the melting of GMS and for homogeneous mixing of the oil phase.

An aqueous phase i.e. distilled water and Tween 80 were mixed in a beaker and were heated to the same temperature as that of the oil phase i.e. 75 ºC. Finally, oil phase was added to the aqueous phase dropwise using a mixer SILENT CRUSHER M (Heidolph, Germany) for 10 minutes at 10,000 rpm and for further 5 minutes at 5,000 rpm until mixture gets cooled down. At 30 ºC, the lemon grass extract was added in the formulation and it was further homogenized by using the SILENT CRUSHER M (Heidolph, Germany) for 5 minutes to obtain

**Table 2.** Composition of different trialed formulations

| | Composition (% w/w) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | GMS | P.A | B.W | TW.80 | S.O | C.A | Gly | KOH | S.A | L.G.E | Water (Q.S) |
| $T_1$ | 1 | 1 | - | 1 | - | - | - | - | - | 5 | 100 |
| $T_2$ | 2 | 1 | - | 1 | - | - | - | - | - | 5 | 100 |
| $T_3$ | 2 | 2 | - | 1 | - | - | - | - | - | 5 | 100 |
| $T_4$ | 1 | 2 | - | | - | - | - | - | - | 5 | 100 |
| $T_5$ | 2 | 2 | - | - | - | - | - | - | - | 5 | 100 |
| $T_6$ | 5 | 2 | 1 | - | - | - | - | - | - | 5 | 100 |
| $T_7$ | 5 | 2 | - | - | - | - | - | - | - | 5 | 100 |
| $T_8$ | - | - | - | - | - | 2 | 2 | 0.8 | 8 | 5 | 100 |
| $T_9$ | - | - | 0.5 | - | - | - | 2 | 0.8 | 8 | 5 | 100 |
| $T_{10}$ | - | - | - | - | - | 2 | 2 | 0.8 | 6 | 5 | 100 |
| $T_{11}$ | - | - | - | - | - | 2 | 2 | 0.8 | 4 | 5 | 100 |
| $T_{12}$ | 2 | - | - | 2 | 5 | - | - | - | - | 5 | 100 |
| $T_{13}$ | 5 | - | - | 2 | 10 | - | - | - | - | 5 | 100 |

GMS= Glyceryl mono stearate.
P. A= Palmitic acid.
TW.80= Tween 80.
C.A= Cetyl Alcohol.
KOH= Potassium hydroxide.
L.G.E= Lemon Grass Extract
Q.S = Quantum satis
B. W= Bees wax.
S. O= Silicone oil.
Gly= Glycerine.
S. A= Stearic acid.

a stable formulation of the desired consistency. Similar procedure was followed for different trial formulations.

### 2.9 Emulsion type confirmation

For the confirmation of O/W emulsion 1 drop of emulsion was mixed with 1 drop of distilled water on a slide and mixing was done using a tooth pick then a small quantity of amaranth was added to the slide and a smear was made and then slide was observed under microscope (Libomed, USA). As amaranth is miscible in water so the continuous phase would gain the color of amaranth i.e. red and dispersed phase i.e. oil droplets were seen against red background. This confirms the emulsion type i.e. O/W type.

### 2.10 Short term stability (Centrifugation tests)

Centrifugal tests were employed for emulsions right after their preparation. Those tests were repeated after 1 day and 7 days of storage at the temperatures of 25° C and 40° C. They were subjected to centrifugation at 5,000 rpm and 25° C for 10 min by placing 10 g of sample in centrifugal tubes of centrifuge machine (Model: 800) [22].
Relative Amount (%) = Vo / V x 100
$V_o$= Volume Instability Phenomenon
V= Total Volume

### 2.11 Thermal stress tests

Stability tests were done at different settings for emulsions to check the effect of these conditions on the stability of emulsions. These tests were performed on samples kept in refrigerator (Dawlence model # 9122M, Pakistan) at 4° C±2°C, room temperature at 25° C ± 2°C and in thermal electric thermostatic drying oven (DHG-9202, SANFA, China) at 40° C ± 2°C. Phase separation and liquefaction of emulsions were observed at various time intervals during 28 days. pH values of freshly prepared emulsions and emulsions kept at different conditions were determined by a digital pH-Meter . The pH tests were repeated after 24 hours, 3 days, 7 days, 14 days, 21 days and 28 days

of preparation [22]. Creaming property for 5 ml of lotion was determined by keeping it at 4° C ± 2°C, 25° C ± 2°C and 40° C ± 2°C for a period of 28 days and separation of solid and liquid phase was also observed.

### 2.12 Rheological studies

Since 1960's rheological measurement has been becoming progressively essential to illustrate the "consistency" of semisolid gels, ointments and creams, ("so-called complex or structural fluids"), in a meaningful fashion [23]. Rheological profiles are now mandatory in numerous pharmaceutical and cosmetics industries. DV-III-Ultra Rheometer (Brookfield Engineering Lab) was used to check the rheological profile of the lemongrass formulation i.e. sunscreen lotion.

### 2.13 Freeze thaw test

Stability testing was done by using freeze thaw cycling method. The temperature was altered every 24 hours between 25°C and -5°C for ten cycles and samples were observed for physical stability. To check the physical stability of emulsion, its color, pH, liquefaction, phase separation and creamy texture were observed at the end of each cycle for 10 cycles.

## 3. RESULTS AND DISCUSSION

### 3.1 2, 2-Diphenyl-2picrylhydrazyl radical scavenging capacity assay

DPPH radical scavenging capacity assay was done to check the antioxidant potential of lemongrass extract. Ascorbic acid (vitamin C) was taken as standard. Assay indicates the antioxidant capacity of lemongrass extract to be 94.20% and that of vitamin C was 92% as shown in **Table. 3**. The results indicate that lemongrass extract possesses slightly more antioxidant potential as compared to the standard i.e. vitamin C.

### 3.2 Selection of the desired formulation:

For the selection of desired formulation, selection criteria were developed, according to which the most stable and suitable sunscreen formulation was selected for further study as shown in **Table 4**. Thirteen (13) trials were performed and for the selection of desired one the following parameters were set; phase separation, sedimentation/

**Table 3.** Antioxidant activity of lemongrass extract in comparison with vitamin C

| Compositions | Antioxidant activity (%) |
|---|---|
| Lemongrass Extract | 94.2 |
| Ascorbic Acid (Vitamin C) | 92 |

**Table 4.** Selection criteria of desired formulation

| | Phase separation | Sedimentation /creaming | Liquefaction | Consistency | Microbial Growth | Phase Inversion |
|---|---|---|---|---|---|---|
| $T_1$ | ✓ | ✓ | ✓ | Watery | × | × |
| $T_2$ | ✓ | ✓ | ✓ | Watery | × | × |
| $T_3$ | ✓ | × | ✓ | Watery | × | × |
| $T_4$ | × | × | × | Watery | × | × |
| $T_5$ | ✓ | ✓ | ✓ | Cream | × | × |
| $T_6$ | × | × | × | Cream | ✓ | × |
| $T_7$ | × | × | × | Cream | ✓ | × |
| $T_8$ | × | × | × | Thick cream | × | × |
| $T_9$ | × | × | ✓ | Cream | × | × |
| $T_{10}$ | × | × | × | Cream | × | × |
| $T_{11}$ | × | × | × | Cream | ✓ | × |
| $T_{12}$ | × | × | × | Watery | × | ✓ |
| $T_{13}$ | × | × | × | Lotion | × | × |

creaming, liquefaction, consistency, microbial growth and phase inversion. $T_1$ (trial 1) and $T_2$ (trial 2) were rejected due to its watery consistency, phase separation, sedimentation and liquefaction. In $T_3$ (trial3), phase separation and liquefaction was observed and its consistency was watery. There was no physical instability observed in $T_4$ (trial 4), $T_8$ (trial 8) and $T_{10}$ (trial 10) but these were rejected due to their poor consistency - $T_4$ was watery; $T_8$ and $T_{10}$ were of creamy consistency. $T_5$ (trial 5) was rejected due to phase separation, sedimentation, liquefaction and creamy consistency. In $T_6$ (trial 6) and $T_7$ (trial 7), there was visible microbial growth along with creamy consistency, so these were also rejected. In $T_9$ (trial 9), liquefaction was observed along with creamy texture so it was also excluded. The rejection of $T_{11}$ (trial 11) was due to microbial growth and creamy consistency while that of $T_{12}$ (trial 12) was of watery consistency along with phase inversion. $T_{13}$ (trial 13) was selected because of its desired characteristics, there was no physical instability observed in this trial also it had the desired consistency of a lotion.

Creaming/sedimentation leads to phase separation and often occurs due to density differences amongst the two phases under the effect of gravity [24]. From the very beginning, the temperature and time processes begin to contribute to emulsion separation, leading to a decrease in viscosity, and as a consequence liquefaction occurs [25].

### 3.3 Characterization tests

The pH of human skin usually varies from 4.5 to 6.0. So, it is essential for a formulation that its pH value should lie within this range [26]. Determining the pH value is critical for assessing the emulsions' stability. In fact, pH fluctuations show the incidence of chemical reactions that can indicate the quality of the final formulation [27]. That is why pH is included in all the stability studies such as centrifugation, freeze thaw test and thermal stress test.

### 3.4 Short-term stability (Centrifugation tests)

Apparently, there was no effect of centrifugation on the emulsion. No phase separation, liquefaction, sedimentation was observed as shown in **Table 5**. This was probably due to the appropriate homogenization speed during emulsion formulation which might have prevented the breakage of the formulations during testing [28].

**Table 5.** Short-term stability of lotion (centrifugation test)

| | **Stability of lotion at different time intervals** | | | |
|---|---|---|---|---|
| | **At day 1** | | **At day 28** | |
| | **25° C** | **40° C** | **25° C** | **40° C** |
| **Liquefaction** | × | × | × | × |
| **Phase separation** | × | × | × | × |
| **Creaming** | × | × | × | × |

×=no change.

**Table 6.** Thermal stability of lotion

| | **Stability of lotion at time intervals** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1st day** | | | **7th day** | | | **14th day** | | | **21st day** | | | **28th day** | | |
| | **4°C** | **25°** | **40°** | **4°** | **25°** | **40°** | **4°** | **25°** | **40°** | **4°** | **25°** | **40°** | **4°C** | **25°** | **40°C** |
| **Co** | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C |
| **pH** | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.4 | 5.5 | 5.5 | 5.3 | 5.5 | 5.5 | 5.3 | 5.5 | 5.5 | 5.24 |
| **L** | × | × | × | × | × | × | × | × | × | × | × | × | × | × | × |
| **P.S** | × | × | × | × | × | × | × | × | × | × | × | × | × | × | × |
| **Cr** | × | × | × | × | × | × | × | × | × | × | × | × | × | × | × |

**C =** Cream**, Co** = Color**, Cr**=Creaming**, L**=Liquefaction,
**P. S**= Phase separation**,** ×= no change

### 3.5 Thermal stress tests

There were no visible signs of lotion instability after thermal stress test as shown in **Table 6**. No changes were observed in any of the parameter except pH and the possible reason was explained earlier that it could be due to destabilization of emulsion caused by hydrolysis process at high temperature [27].

### 3.6 Rheological studies

Rheological behavior is adjusted as per need and purpose of usage of a particular cosmetic product. For example, body lotions need a certain yield stress (or high viscosity) at rest to stay in the hands of the user while being taken out of the bottle. However, a consequent shear thinning manner (or low viscosity at high shear) is essential for easiness of spreading and applying the lotion onto the skin. The low viscosity at high shear is also important for lotion to form a uniform thin layer that will more easily penetrate the skin and help the skin absorb the active ingredients without feeling oleaginous or gluey [29].

The rheological results obtained by using rheometer ( DV-111-ultra Rheometer, Brookfield Engineering Lab) indicate the non-Newtonian behavior of the formulation; it has pseudo-plastic and shear thinning behavior as shown in **Table 7** which indicates that it can be handled without difficulty while its application on skin.

### 3.7 Freeze thaw Test

There was no significant change in the formulation stability as shown by the results of this test in the **Table 8**. No changes were observed in the color, liquefaction, phase separation and creaming. However, slight difference was observed in the pH that may be due to the changing temperature but overall the pH change was negligible to have any influence on the stability of the formulation because the pH at the end of the freeze thaw test was 5.18 which lies in the range of skin pH i.e. 4.5 to 6.0 [26] and is not a skin irritating pH [30].

### 3.8 SPF determination

SPF of the extract was determined using UV-visible spectrophotometer (HALO DB-20) and by applying Mansur equation. The absorbance was determined via spectrophotometer at a wavelength range of 290nm to 320nm and then by applying the Mansur equation the SPF was determined. The calculated value of the extract is given in the **Table 9**. For the determination of SPF of the formulation, three different dilutions i.e. 2000µg/ml, 400µg/ml and 80µg/ml were made and SPF values were determined as done for the lemongrass extract. Maximum SPF value was obtained at 2000 µg/ml i.e. 22 and at decreased concentrations, SPF values also showed a decreasing trend as shown in **Table 9**.

**Table 7.** Rheology of Lotion

| NO. | Viscosity (η) (cP) | Speed (RPM) | % Torque (%) | Shear Stress (τ) (D/cm²) | Shear Rate (γ) (1/sec |
|---|---|---|---|---|---|
| 1 | 3.75 | 5.00 | 0.1 | 0.25 | 10.00 |
| 2 | 3.68 | 10.00 | 0.3 | 0.74 | 20.00 |
| 3 | 3.27 | 15.00 | 0.4 | 0.98 | 30.00 |
| 4 | 3.07 | 20.00 | 0.5 | 1.23 | 40.00 |
| 5 | 2.75 | 25.00 | 0.7 | 1.72 | 50.00 |
| 6 | 2.46 | 30.00 | 0.6 | 1.47 | 60.00 |
| 7 | 2.46 | 35.00 | 0.7 | 1.72 | 70.00 |
| 8 | 2.46 | 40.00 | 0.8 | 1.96 | 80.00 |
| 9 | 2.46 | 45.00 | 0.9 | 2.21 | 90.00 |
| 10 | 2.46 | 50.00 | 1.0 | 2.46 | 100.00 |

Rheocalc V2.6 Brookfield Engineering Labs
Math Model: Power Law Consistency Index: 3.95 cP
Flow Index: 0.91 Confidence of Fit: 87.3 %
Spindle: CP41

**Table 8.** Freeze thaw of sunscreen lotion

| Time interval | Temperatures | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | -5°C | | | | | 25°C | | | | |
| | Color | pH | Lique-faction | Phase separation | Cream-ing | Color | pH | Lique-faction | Phase separation | Cream-ing |
| Day1 | - | - | - | - | - | Cream | 5.51 | × | × | × |
| Day2 | Cream | 5.5 | × | × | × | - | - | - | - | - |
| Day3 | - | - | - | - | - | Cream | 5.47 | × | × | × |
| Day4 | Cream | 5.47 | × | × | × | - | - | - | - | - |
| Day5 | - | - | - | - | - | Cream | 5.45 | × | × | × |
| Day6 | Cream | 5.42 | × | × | × | - | - | - | - | - |
| Day7 | - | - | - | - | - | Cream | 5.42 | × | × | × |
| Day8 | Cream | 5.41 | × | × | × | - | - | - | - | - |
| Day9 | - | - | - | - | - | Cream | 5.40 | × | × | × |
| Day10 | Cream | 5.39 | × | × | | - | - | - | - | - |
| Day11 | - | - | - | - | - | Cream | 5.35 | × | × | × |
| Day12 | Cream | 5.33 | × | × | × | - | - | - | - | - |
| Day13 | - | - | - | - | - | Cream | 5.31 | × | × | × |
| Day14 | Cream | 5.28 | × | × | × | - | - | - | - | - |
| Day15 | - | - | - | - | - | Cream | 5.25 | × | × | × |
| Day16 | Cream | 5.23 | × | × | × | - | - | - | - | - |
| Day17 | - | - | - | - | - | Cream | 5.21 | × | × | × |
| Day18 | Cream | 5.20 | × | × | × | - | - | - | - | - |
| Day19 | - | - | - | - | - | Cream | 5.19 | × | × | × |
| Day20 | Cream | 5.18 | × | × | × | - | - | - | - | - |

**Table 9.** SPF value determination of lemongrass extract and sunscreen lotion

| S. No. | Wavelength | EE×I | LEMONGRASS EXTRACT | LEMONGRASS SUNSCREEN | | |
|---|---|---|---|---|---|---|
| | λ (nm) | Normalized | 10,000µg/ml | 2000µg/ml | 400µg/ml | 80µg/ml |
| 1 | 290 | 0.0150 | 0.015 | 0.033 | 0.013 | 0.004 |
| 2 | 295 | 0.0817 | 0.076 | 0.180 | 0.072 | 0.023 |
| 3 | 300 | 0.2874 | 0.253 | 0.638 | 0.252 | 0.080 |
| 4 | 305 | 0.3278 | 0.276 | 0.721 | 0.284 | 0.090 |
| 5 | 310 | 0.1864 | 0.154 | 0.417 | 0.161 | 0.051 |
| 6 | 315 | 0.0839 | 0.080 | 0.188 | 0.072 | 0.023 |
| 7 | 320 | 0.0180 | 0.014 | 0.040 | 0.015 | 0.005 |
| **SPF** | | | 8.5 | **22** | **8.7** | **2.8** |

SPF of the extract was 8.5 at a dilution of 10,000 µg/ ml, while SPF values of formulation were 22, 8.7, and 2.8 at dilutions of 2000 µg / ml, 400 µg / ml, and 80 µg / ml, respectively. The decreasing trend of SPF values observed with decreasing concentration is evident in **Table** 9. Results clearly indicate a dose dependent increase in SPF value and at 2 mg/ml concentration; lemongrass shows good photoprotective ability in *in-vitro* studies. These results are consistent with previous and recent studies in which plant extracts have been explored for photoprotection.

In a study, Calendula oil cream showed good photoprotection ability (SPF = 14.84 ± 0.16) at 5 % concentration of extract [31]. Sri Lankan researchers focused on evaluation of photoprotective activity of some aqueous herbal extracts at a fixed concentration of 1 mg/ml and among the extracts, *Atalantia ceylanica, Hibiscus furcatus, Leucas zeylanica, Mollugo cerviana, Olax zeylanica* and *Ophiorrhiza mungos* have shown SPF value≥25, which were even higher than two commercial photoprotective creams used as reference compounds. Moreover, high antioxidant

activity of extracts was also observed in DPPH assay [32].

A group of researchers evaluated the photoprotective effect of cosmetic formulations containing hydroalcoholic extract of *N. variegata* (Nv-HA). Initially, the phenolic and flavonoid total content of Nv-HA were determined. The photoprotective activity of Nv-HA showed SPF values of 5.43 ± 0.07 and 11.73 ± 0.04 at the concentrations of 0.5% and 1.0% (v/v), respectively. It was also verified that Nv-HA potentiated the photoprotective effect of formulations in a dose dependent manner and formulations remained stable at the end of the study [33].

## 4. CONCLUSION

The *in-vitro* spectrophotometry method was used in this study which is a simple and an easy method for determination of SPF value of sunscreen emulsions. For the determination of antioxidant potential, DPPH radical scavenging capacity assay was performed. The results showed the antioxidant potential of lemongrass extract to be 94.20% and the lemongrass sunscreen lotion to have SPF value of 22. The pH of lotion was found 5.5 which comply with the skin pH. Viscosity profile of lotion indicated good rheology during handling. No phase separation was observed after centrifugation, freeze thaw and thermal stress tests which indicated stability of formulation.

Along with their many beneficial effects and safety, the natural product could become a good, cheap and easily available formulation ingredient in sunscreen products**.**

## 5. ACKNOWLEDGEMENTS

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Research Article

# Sub-Lethal Effect of Waterborne Cadmium Exposure on Glutathione S-Transferase and Total Protein Contents in Liver of Carnivorous Fish, *Wallago attu*

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**Abstract:** This research work was performed to evaluate the glutathione S-transferase activity and total protein contents in liver of carnivorous fish, *Wallago attu* exposed to sub-lethal concentrations (1/3rd, 1/4th and 1/5th) of cadmium for14days. Fish was sampled after 7 days interval. Spectrophotometric method was used to analyze the GST activity and protein contents. Results showed that the exposure of waterborne cadmium significantly ($p<0.05$) increased the GST activity in liver of fish in relation to control. However, protein contents were significantly ($p<0.05$) decreased due to metal exposure. It was also concluded that concentration and duration of exposure greatly influenced GST activity and total protein contents in fish. Regression analyses showed that GST activity had significantly positive relation with cadmium concentrations while protein showed significantly negative relationship with concentration.

**Keywords:** heavy metal, carnivorous fish, Biochemical parameters

## 1. INTRODUCTION

Today, human beings have faced a major problem of environmental pollution including land, water and air pollution. Among these, water pollution is difficult to be measured as compared to air and land and air pollution [1]. The behavior of toxicants in water envisages biological response of an aquatic ecosystem. Different toxicants act through their toxicity, fate and specific nature while the response of biological system involves adaptations, defense, stress response and recuperation [2]. A number of organic and inorganic substances include hazardous wastes, textile dyes, phenol, petroleum and explosive products and heavy metals are the major sources of pollution.

Heavy metals are the chief constituent of inorganic pollutants in aquatic system [2-4].Some metals are indispensable; even that metals are lethal at higher concentrations. Metals can produce oxidative stress by stimulating the formation of reactive oxygen species (ROS), and can substitute crucial metals in enzymes or pigments disrupting their function [2].Contamination of heavy metals may have negative impact on aquatic environment and also on the variety of water life [5-7].

Cadmium (Cd) is a dispensable heavy metal, known as one of the major pollutants in natural water [8]. According to Hayat et al. [9] *Wallago attu* showed decreased growth after sub-lethal exposure to waterborne Cd for long duration (20-day). At higher concentration Cd rapidly causes deficiency of calcium and low blood hemoglobin in fish [10] and can also cause internal injuries in fish. Several studies also reported that Cd may be linked with oxidative damage for the generation of ROS [11-12]. Generally, antioxidant defense system contains superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase. These enzymes are present in all body organs of vertebrates, but liver showed higher activity, a major organ for detoxification of xenobiotic [13]. The glutathione *S*-transferases (GSTs) belongs to phase II detoxification enzyme, which can

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minimize the toxicity of a variety of endogenous and exogenous compounds by facilitating nucleophilic attack by reduced glutathione (GSH) [14]. The GSTs enzymes are now increasingly evaluated in most of aquatic studies as sensitive biomarkers of exposure to environmental toxicants [15].Protein modification as a consequence of free radicals is also an important parameter for evaluating the oxidative stress [16-17].

*Wallago attu* commonly known as "helicopter" is a freshwater carnivorous fish belongs to family Siluridae. It is locally called Mulli**.** It is experiencing population decline when migrate through urban and agricultural water courses have persistent water pollutants such as pesticides and metals. Therefore in the present study glutathione S-transferase activity and total protein contents in the liver of fish *Wallago attu* under cadmium exposure was evaluated.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Design

Fingerlings of carnivorous fish, *Wallago attu* were selected for this research work. The freshwater fish, *W. attu* were collected from their natural breeding ground and transported to the laboratory of University of Agriculture, Faisalabad. Prior to experiment *W. attu* fingerlings were kept in cemented tank to acclimatized laboratory conditions for two weeks. After acclimatization period, fish were transferred to 70-L glass aquarium. Each aquarium contained a group (n=10) fishes. The total hardness (225mg$L^{-1}$), pH (7.25) and temperature (30$^{\circ}$C) of water were kept constant throughout study period. However, calcium, magnesium, sodium, potassium, total ammonia, carbondioxide and electrical conductivity were also measured on daily basis by following the method described in A.P.H.A. [18]. Continuous air was supplied to all the test and control medium with an air pump through capillary system. The chemically pure chloride salt of cadmium was used to prepare stock solution. The 96-$LC_{50}$of cadmium for *W. attu* was reported as 32.96 $mgL^{-1}$ by Batool et al. [19].Fish were exposed to sub-lethal concentrations viz. 1/3$^{rd}$, 1/4$^{th}$and 1/5$^{th}$ of $LC_{50}$ for 14 days and fish sampling was done after 7 and 14 days.

### 2.2. Preparation of Homogenate

The enzyme glutathione S-transferase was isolated from the liver of *Wallago attu.* The organ was weighed i.e. liver. To remove the RBCs the dissected organ was rinsed with 50m M Tris HCL buffer of PH 7.4 and containing 0.2 M sucrose 4 times greater than the weight of organ, 1.4 and homogenized for 15 minutes in cold buffer (1:4 w/v) using a pestle and mortar. After homogenization, organ homogenates were centrifuged for 15 minutes at 10,000 rpm and 4$^{0}$C. After centrifugation process, clear supernatants were stored at -80$^{0}$C for enzyme assay while residue was discarded.

### 2.3 GST Assay

Activity of GST was measured by following the method of Habig and Jakoby [20] at 340nm against the reagent blank on spectrophotometer after interval of 1-minute.

### 2.4 Total Protein Contents

To estimate total protein content of samples Biuret method [21] was used.

### 2.5 Statistical Analysis

After the calculation of enzyme activity, obtained data were subjected to statistical analyses by using the Factorial experiments with three replicates. The value of $p<0.05$ was considered statistically significant. Regression analyses were also performed to find-out possible relationships between Peroxidase activity and exposure duration.

## 3. RESULTS

Results showed that the exposure of cadmium significantly increased the GST activity in the liver of *W. attu* in relation to control. Comparison among different concentrations of cadmium showed that maximum GST activity was observed under 1/3$^{rd}$ concentration followed by that of 1/4$^{th}$ and 1/5$^{th}$ (Table 1). Total protein contents were decreased in liver of exposed fish than that of control (Table 2). It was observed that protein contents were decreased in following order:1/3$^{rd}$>1/4$^{th}$>1/5$^{th}$. Regression equation showed that GST activity had significantly positive relation with cadmium

**Table 1.** GST (U/mL) activity in liver of *W. attu* exposed to sub-lethal concentrations of cadmium

| Duration of Exposure | Control | Treated | | |
|---|---|---|---|---|
| | | 1/5th of $LC_{50}$ | 1/4th of $LC_{50}$ | 1/3rd of $LC_{50}$ |
| **7 days** | 550.33±1.41d | 575.67±0.86c | 599.88±0.83b | 644.92±0.82a |
| **14 days** | 550.67±13.01d | 592.37±0.77c | 650.33±0.50b | 756.66±0.50a |

Means with similar letters in a single row are statistically similar at $p<0.05$.

**Table 2.** Total protein contents in liver of *W. attu* exposed to sub-lethal concentrations of cadmium

| Duration of Exposure | Control | Treated | | |
|---|---|---|---|---|
| | | 1/5th of $LC_{50}$ | 1/4th of $LC_{50}$ | 1/3rd of $LC_{50}$ |
| **7 days** | 8.67±0.77a | 7.88±0.94b | 6.61±0.76c | 5.78±0.29cd |
| **14 days** | 8.64±0.67a | 6.42±0.50b | 6.03±0.68c | 4.19±0.67d |

Means with similar letters in a single row are statistically similar at $p<0.05$.

**Table 3.** Relationship between biochemical parameters of *W. attu* and lead concentrations

| Biochemical Parameters | Regression Equation | SE | r | $R^2$ |
|---|---|---|---|---|
| **GST Activity** | 407 + 26.7 *Concentration | 0.7226 | 0.999 | 0.999 |
| **Total Protein** | 10.4 - 0.491 **Concentration | 0.004679 | 0.999 | 0.999 |

SE=Standard Error; r= Multiple Regression Coefficient; Coefficient of Determination;
**=Highly significant at $p<0.01$, *= Significant at $p<0.05$.

concentrations while protein showed significantly negative relationship with concentration (Table 3).

## 4. DISCUSSION

Oxidative stress occur when there is an inequality between the generation of reactive oxygen species (ROS) and the ability of cell to diminish ROS. This inequality may be due to elevated level of ROS, a decline in enzymatic activities or both. Numerous ecological pollutants like heavy metals are recognized as inducer of oxidative stress [22]. Oxidative stress biomarker such as biochemical parameters include change in antioxidant enzyme activity and protein contents as indicated by *W. attu* exposed to cadmium.

Glutathione S-transferase (GST) enzyme is a constituent of antioxidant enzyme performing a key function in xenobiotic detoxification in the cell. In current work, GST activity was significantly increased in liver of cadmium exposed fish in relation to control.

Our results are supported by Espinoza et al [23] who reported the significant induction in GST expression of *Ictalurus melas* liver after exposure to heavy metal cadmium. Significant elevation in hepatic GST activity of *Oreochromis niloticus* exposed to cadmium was observed by Zirong and Shijun [24]. Exposure of cadmium significantly increased the GST activity in puffer fish (25).Lopes et al [26] investigated augmented GST in the liver of freshwater fish, *Leuciscus alburnoides* captured from the cadmium polluted area. Bozcaarmutlu et al [27] determined the higher GST activity in the liver of fish captured from polluted site than the reference site. Sub-lethal concentration of cadmium caused induction in liver GST activity of *Oreochromis mossambicus* [28]. Vinodhini and Narayanan [29] also reported the significant increase in liver GST activity of common carp exposed to heavy metal solutions. Elevated level of GST in *Oreochromis niloticus* under sub-lethal levels of cadmium was reported by EL-Gazzaret al [30].

Protein is an important structural and functional part of the cells performed various crucial functions such as they may serve as a major energy source under sub-lethal stress and also key

sourcesof nitrogen metabolism [31].Our findings are also supported by Faheem et al [32]reported the decreased protein contents in liver of cadmium exposed fish *Oreochromis niloticus*. Tantarpale [33] reported a decline in total protein contents of fish *Channa striata* exposed to toxicants (pesticides), *C. Fasciatus* (34), *Tor putitora* [35], Nile tilapia [36], *Labeo rohita* [37].

## 5. CONCLUSION

The findings of this study demonstrated that exposure of heavy metal cadmium at sub-lethal concentration can change the biochemical parameters like antioxidant enzyme and protein contents of fish. Furthermore, these biochemical parameters will be helpful in determine the toxic effect of metals on fish.

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Research Article

# Impact of Eutrophication on Shallow Marine Water near Karachi Coast, Pakistan

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**Abstract:** The coastal belt of Karachi, Pakistan was studied to estimate the nutritional levels of shallow seawater. It is investigated to study the localized eutrophication phenomena in the coastal region of Ibrahim Haideri and Karachi Port Trust at Kemari. It was found that the coastal regions of Port Qasim, Clifton and Hawke's Bay Beach when compared with the coastal regions of Ibrahim Haideri and Karachi Port Trust Kemari are more impacted with anthropogenic contamination. Over presence of reactive nitrogen and other nutrients were the indication of disposal of solid waste, sewage effluent and industrial effluents. Oxygen depletion due to localized eutrophication causes too much death of biotic components of marine ecosystems that in turn sink to the bottom of shallow water of Ibrahim Haideri and coastal areas of Karachi Port Trust. This phenomenon increases the microbial decomposition, which in turn decreases the average oxygen level to 6.71 mg/L to 6.99 mg/L at Coastal region of Ibrahim Haideri and Karachi Port Trust respectively. The ammonia, nitrogen was calculated as average 22.48 ppm in the seawater samples collected from Ibrahim Haideri and Karachi Port Trust. The average of total phosphorus in the collected from both these coastal regions were estimated as 1.164 mg/L, with high TDS and TSS values, i.e. average 51598.5 mg/L and 862.75 mg/L in the seawater.

**Keywords:** Echo System, shallow marine water, coastal belt, oxygen level, phosphorus level, TDS

## 1. INTRODUCTION

Recent human developments have influenced all main aquatic environments and their activities have replaced the natural composition of marine water and coastal ecosystem. One main reason behind this fact is accelerated aggrandizement of agricultural activities [1]. However, the global formation of soil fertilizing chemicals are more impacted on aquatic systems and it is estimated that it releases more than 510 million metric tons of reactive nitrogen in 1950, this can increase 135 million metric tons of reactive nitrogen by the year 2030 [2]. The more amount of reactive nitrogen is added to agricultural fields by introducing domestic animal manure. Same is the case as little, but ecologically more important part of agricultural phosphorus is introduced from land surface of the earth's crust to receiving water bodies of lakes, rivers and then finally mixed into the marine water. Atmospheric phosphorus is also loaded to surface water bodies and eventually get way to marine water [3-6]. Eutrophic water is water of lake, river or sea where abundant nutrients in the form of nitrogen and phosphorus found. However, aquatic eutrophication alters the normal chemical composition of water bodies, so in other words

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eutrophication is an enrichment of water bodies with different nutrients, especially compounds of nitrogen (reactive nitrogen) and phosphorus. This may cause unwanted change in ecology or natural inhabitants of the aquatic environment and can alter the quality of water and its color, eutrophication causes accelerated growth of algal blooms over water.

The main anthropogenic sources via enrichment of nutrient occurs (i.e., runoff, erosion and leaching from fertilized agricultural areas, and sewage from cities and industrial wastewater). These nutrient inputs have profound effects on the natural manifestation of acquiring water bodies [7, 8]. The visible reaction of marine ecosystem by eutrophication is the greening of top of the water column in the shape of development of algal growth (i.e., algal mat) and vegetation over coastal regions which develop a straight response to nutrient enrichment. Although the most serious impact of eutrophication is unnoticed decrease in dissolved oxygen (DO) concentration (of marine water, which creates deaths of algae and plants.

This phenomenon adds too much pure organic biomolecules to seafloor and provides good favorable conditions for microbial decomposition. A large amount of nutrients collected from land surface via different routes to streams and rivers finally finds their direction toward marine water; eventually estuaries acquire a high level of nutrient inputs per unit surface area [9]. World's greater than half the human population resides under 60 km of coastal environment, in this way 90% or greater world's fisheries depend in one way or another on estuarine and near shore habitats [10].

### 1.1 Description of Arabian Sea and Pakistan coastline

Arabian Gulf, Gulf of Oman and Arabian Sea, all combined together to form Arabian Seas, since old time it constitutes most important trade zone [11, 12, 13]. Arabian Sea is the northern region of the Indian Ocean linked to the north by Pakistan and Iran, as on the west by northeastern Somalia and Arabian Peninsula, and on east by India. Recently pollutants that most severely impacted on this costal environment and its physical and biological features are harmful algal blooms (HABs). However, since the last period of time these red tides or HABs found more frequent over the surface of Arabian Sea and transported from one coastal zone to another. This algal blooming is a more serious problem for the region [14]. The coastal regions of the Arabian Sea are undergoing high industrial growth along with urban developing projects that further increase the conditions of extreme destruction of naturally under stressed marine ecosystems. Coastal communities of marine environment are under the impact of untreated industrial effluent, brine effluent, ports and refinery waste, oil discharge and domestic discharge. The geographic location of Pakistan is such that it is facing directly the Arabian Gulf, bordering Iran, India, close to Oman along with landlocked central Asian countries.

Pakistan provides better facilities to benefit from its geo strategic position. Increasing levels of destruction of the coastal resources of the country are a very critical issue, and immediate response is needed. Pakistan coastline is about 990 km long having an adjacent coastal zone of 240,000 square km in the Northern Arabian Sea. This area was explored and, thus, new ports, tourist resorts and industrial sites were established as a part of enhancing national economic activity. Coastal location of Pakistan consists of two distinct units, the passive margin of Sindh coastline covers 370 km and other active margin of the Balochistan coastline include and covers 760 km. Pakistan as its geographic position is very important so it provides a vital trade route. Pakistan provides main oil supply routes from the Persian Gulf; however, this increases the chances of oil spillage in the sea. This whole coastal area of Pakistan supports living and nonliving resources. The living resources comprise mangrove ecosystems in Sindh and Balochistan coastline that link Indus delta; it is worth noting that the mangrove ecosystem in this region is the sixth longest in the world.

The commercial significance of the sea that comes in Pakistan's jurisdiction is because of the fact that it holds 350 different species of fish that are consumed locally and exported massively throughout the world. Coastal ecological system of Pakistan is a zone of naturally equipped resource region, which provides economic material and services as well, material and services both marketed as fish, shellfish and non-marketed like mangroves

commonly used in medicine and provide nursery areas for juvenile fish it provides buffers against storm surges [15].

### 1.2 Components of marine ecosystem of Arabian Sea

Human communities of the coastal regions of Arabian Sea depend upon sea on the bases of economic, culture and social aspects. The ecosystems of sea grass beds, coral reefs, mangrove swamps, and mudflats play a role in productivity and constitute rich marine coastal resources of Arabian Sea [16]. The producers of the Arabian Sea ecosystem are seagrass beds that function ecologically and economically [17]. Ecologically, these components of ecosystem bring food material, they provide feed ground for various species that are under threat of environmental conditions of Arabian Sea, and these species include turtle and dugongs [18, 19]. On economical bases, they provide important nursery to the sea floor for shrimps, pearl oysters and various different animals of Arabian Sea [20]. These seagrass species have ability to tolerate the extreme saline conditions along with increased temperature in the Arabian Sea. They have the ability to tolerate the salinity 70 psu in summer, and the temperature exceeds 31ºC.

An ecological system of corals brings different functions, they maintain renewable resources of seafood genetic, biological and habitat diversity, recreational values, and economic benefits such as utilizing reefs for creating the land. Coral reefs are characterized by biological diversity and high levels of productivity. They provide high range of habitats for different reef species and fish. Coral reefs are supporting habitats for fishes. Coral growth is visible along many regions of the Arabian Sea; nice proliferation is observed on offshore shoals. Fringe corals mostly found in coastal regions of UAE, Qatar, Saudi Arabia and Bahrain [21].

Arabian Sea provides unfavorable conditions for growth of coral reefs and this is significantly because of the high temperature, high saline water and other physical conditions [22]. Even under those adverse environmental conditions, corals of Arabian Sea have shown resilience and vitality. Recently corals of Arabian Sea exposed to severe temperature as compared to corals of other regions of the world were found to have been tolerant of the thermal environment and that is the reason of its survival [23]. This is a noteworthy fact which draws the regional and international attention to use the Arabian Sea as a model ecosystem to find out environmental impacts and climate change in the future [24].

Ecologically, mangroves are a very necessary ecosystem of Arabian Sea; it brings food, cover and nursery areas for different terrestrial and marine fauna. Mangrove ecosystems of Arabian Sea coastal regions supports and provides shelter to a variety of important species of fish, shrimps, turtles, and birds, and contribute a big role in coastal productivity [25]. There is only one species Avicennia marina found dominated along coastal regions of the Arabian Sea. However, osmoregulation and salt secretion allow Avicennia marina to cope with hyper salinity in Arabian Sea [26]. The sedimentary nature of the Arabian Sea is high, so sand and mud substrata are the most widespread habitats occur along the coastline of the Arabian Sea. These habitats are more favorable areas of mangroves to colonize costal margins and to maintain ecology, these also facilitate algal growth and cyanobacteria, these act as primary producers of food chain. Subtidal and tidal muddy habitats are richer in microbenthic assemblages all these produce huge and constitute very differing marine ecosystem in the Arabian Sea.

### 1.3 Anthropogenic activities

Since the last few decades, anthropogenic activities have profoundly impacted on world's coastal ecosystems by elevation in chemical nutrition levels in coastal regions, and in the same fashion it is contributing an increase in nutrient loading in the Arabian Sea [27]. This may cause serious impact on aquatic ecosystems by increased growth of HABs over Arabian Sea regions [28].

### 1.4 Red tide events and their impacts

The first event of HABs occurred in the history of Arabian Sea was recorded in 1908 along the Indian coastline where the outbreak extended from the Malabar coast towards Laccadive Islands, as this area of Arabian Sea is impacted through India that is rich in phosphate nutrient enrichment. The impact of this was so high that a significant rise in

fish mortality was observed [29].

### 1.5 Impact of Eutrophication

The most common impact of eutrophication is the development of algal bloom, in some instances, this transformation has been accompanied by the appearance and persistence of harmful algal blooms (HABs) to the extent that it has recently been suggested that nutritionally enriched coastal and offshore waters are experiencing an epidemic of harmful phytoplankton blooms [30]. The toxic material of toxic algal blooms (HABs) has the tendency of bio-concentration throughout the food chain. Hence, human population almost occupies a large position in this food chain become easily susceptible to potential impacts of accumulated toxic material [31, 32].

The risk of food poisoning and other gastric infections increase in this way, which is directly connected to sea food ingestion. Marine biologists have investigated that there are two strains of Noctiluca found common, i.e. red coloring and green coloring strains. Sweeney was first ever person who has used term Green Noctiluca. The red water coloration due to algal strain *Noctiluca scitillans* is one of the common reports of this phenomenon in North Arabian Sea [33]. This phenomenon creates very long red color streaking over the surface of seawater investigated in North Arabian Sea [34, 35]. Algal growth with blooming over seawater either toxic or nontoxic cause reduced oxygen level (Hypoxia) at the site of occurrence, which results in a high rate of deaths in seawater, this situation impacted on recreation, fish trade, tourism and health of coastal human communities due to transformation of infectious material into the human food chain [36].

### 1.6 Fate of Microorganisms in Eutrophicated marine environment

In many ecological processes of marine ecosystem, microbiological activities are playing very important role. It is the functions of microbial communities to maintain essential role for biogeochemical cycles, which stand for need to conserve life on earth globe [37]. In marine coastal environmental ecosystems microbial communities contribute natural territory for a range of microbial pathogens such as bacteria, viruses and parasites. Some pathogenic microorganisms inhabit the water and some live adhering to abiotic particles of ecosystem or live inside the bodies of large animals in marine water. The huge range of these animals and greater concentrations of microbial communities within coastal water indicate that the water or even seafood may be infected by organisms associated with human waste [38, 39]. Ingesting semi cooked seafood, which is contaminated with parasites of eutrophicated coastal water, increases the risk of parasitic infections, like anisakids and cestodes, which is found in shellfish [40]. In addition, many research findings have provided information that human pathogens originate from marine environment often transfer infections into the marine mammals exposed to polluted water as like parasitic infection giardiasis and other papilloma virus linked infections with brucellosis that is a bacterial infection [41, 42].

## 2. MATERIALS & METHODS

The research study was designed to understand eutrophication phenomenon of coastal aquatic environment and its impacts on coastal water and coastal ecosystem of Arabian Sea in Karachi, Pakistan. The research material required to undertake research on coastal aquatic environment to check impacts of eutrophication on marine ecosystems of the coastal environment of Arabian Sea in Karachi Pakistan was shallow seawater samples taken from various zones as shown in Fig. 1.

Five principal locations of the Karachi coastal belt were chosen for sampling and field observation survey depending upon the individual coastal location. All water samples were collected according to Grab water sampling technique, and were analyzed qualitatively and quantitatively. Sampling regions are depicted in Fig. 1. Samples were collected during different time intervals throughout this study in two phases.

Different samples from five different locations were collected during retreating climate of the year 2015, in order to check eutrophication level through variation and elevation in temperature, pH, conductivity, color, turbidity, dissolved oxygen, total nitrogen, total phosphorous, the total dissolved solids and total suspended solids of the seawater.

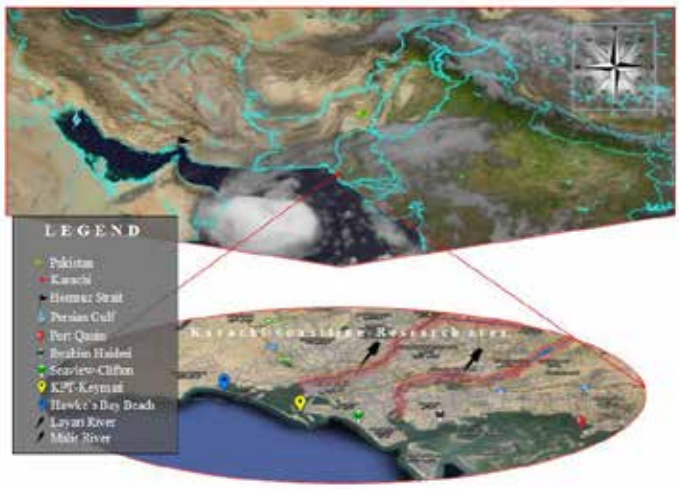
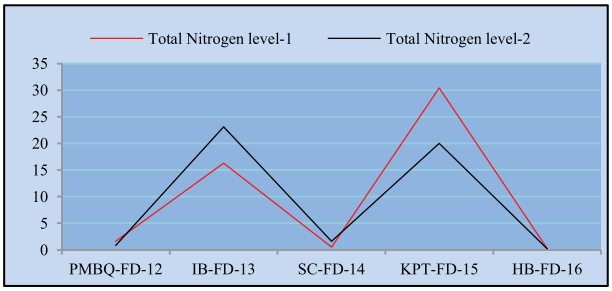
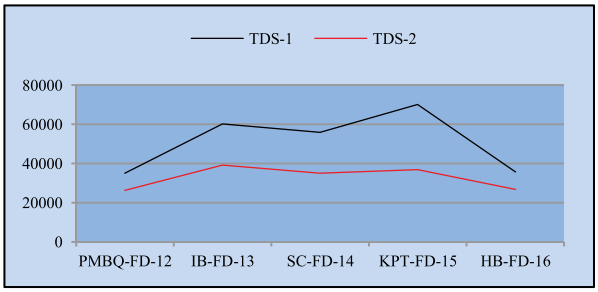


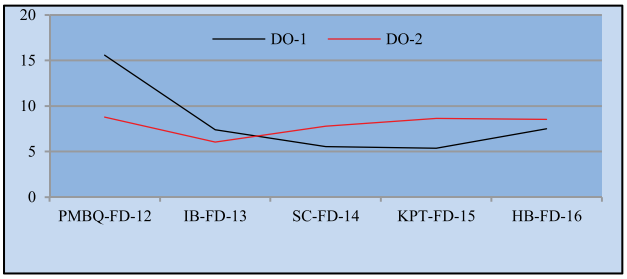

**Fig. 1.** Geographic map showing Arabian Sea and study regions of the Arabian Sea in Karachi coastline Pakistan



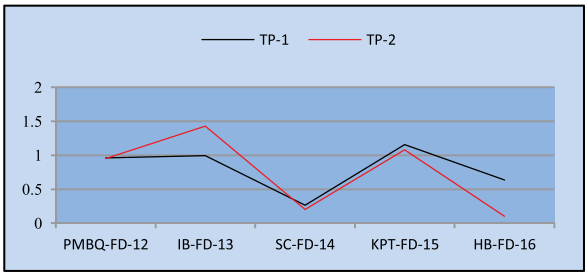

(a) Total Nitrogen level of Karachi coastal zones




(b) Total dissolved solids of Karachi coastal Zones




( c) Dissolved Oxygen level of Karachi coastal zones




(d) Total Phosphorus level of Karachi coastal zones

**Fig. 2.** The levels of total nitrogen, TDS, dissolved oxygen and total phosphorous at various locations

Temperature, pH, conductivity and dissolved oxygen measurement of different coastal zones was recorded immediately after sampling through HACH Sens-ion 156 pH/Conductivity meter, by using pH electrode (i.e. 59005-52), conductivity probe (i.e. 19604-50) and dissolved oxygen probe (i.e. 53013-50). Color of seawater samples was estimated by Platinum Cobalt Standard Method 8025. Turbidity of seawater samples was measured according to International Standardization Organization Method 7027 (ISO Method 7027). Total dissolved solids of seawater samples were estimated through APHA method 2540 C. Total suspended solids of seawater sample was estimated according to APHA method 2540 D. Total phosphorous of seawater samples was examined according to USEPA with acid per sulfate digestion method 8190. Total nitrogen of seawater samples was analyzed according to Nitrogen, Ammonia salicylate method 8155.

The routes of nutrient enrichment of coastal belt of Karachi were identified during the research field survey. It was clear from the survey that disposal of Karachi city's domestic waste (i.e., high in organic constituents), untreated sewage (i.e., high in ammonia, nitrates and phosphates), and disposal of untreated industrial effluent (i.e., rich in various nutrients) via Lyari river and Malir river runs to the seawater of the Karachi coastal belt causes the elevation of nutrient level of seawater.

## 3. RESULTS AND DISCUSSION

Sewage discharges were found to be contaminated with various chemicals and pathogenic microorganism, bioaccumulation and bio-magnification with virulent microorganisms and their toxins potentially impacted on quality of seafood, which in turn pose a severe risk to human health as well. Due to localized eutrophication, suspended and dissolved constituents of coastal water become increased as these contaminating factors were impacted on the natural chemical composition of seawater that leads elevation of seawater Temperature and high TDS and TSS level of the coastal region's seawater, this phenomenon impacted on the physiological characteristics of components of the ecosystem (i.e., growth of mangroves swamps & Seagrass), their diversity and spatial distribution and pathological impacts on marine biotic ecosystems (i.e., fish & shellfish). Although increased nutrient level of seawater estimated due to increased trade activities, frequent shipping introduces additional nutrient factors into the seawater that further increase the nutrient enrichment level of coastal water. Coastal developments also increase the artificial nutrient level of seawater. All of these, thus, lead to the deterioration of normal ecological components of the marine environment. In Karachi city, there are more than 700 small and large industrial units, these are classified into different industrial zones like Sindh industrial trading estate in North Karachi, Landhi industrial trading estate in east Karachi and Korangi industrial area in south Karachi. A significant amount of nutrient dense liquid from all these industrial regions enriches the coastal belt of Karachi which causes localized eutrophication of different coastal regions. Although different contaminating sources play a major contributory role in nutrient enhancement of coastal regions, hence provide nutrient factors for biological stressors of the marine ecosystem such as invasive species and algal blooms, these play further roles in the degradation of marine ecosystems, fish mortalities and dislocation of coastal communities of fish species and other marine animals in different areas of Karachi coast. Localized Eutrophication of Coastal zone of Ibrahim Haideri was eutrophicated with reactive nitrogen by dumping of solid waste collected from different areas of Karachi city and Malir river contaminations.

This localized eutrophication was found elevating the biogeochemical processes of coastal marine water. As the normal nitrogen seawater concentration is almost 0.5 ppm worldwide, However, consecutive ammonia, nitrogen level of the Ibrahim Haideri coastal area was recorded; average ammonia, nitrogen in number of two samples of Ibrahim Haideri coastal seawater was (i.e., 19.7 ppm). Although the average TDS and TSS in number of two seawater samples of this coastal zone was calculated (i.e., TDS=49678 mg/L., & TSS=856 mg/L respectively. The results are presented in Table 1 and Table 2.

The local human communities of this coastal zone depends upon seaborne trade, however enrichment of this coastal zone with organic, inorganic and pathogenic constituents of solid waste and effluent of Malir river negatively impacted on seaborne trade, as the seafood was found heavily contaminated due to eutrophication. Although bioaccumulation and bio magnification of seafood with toxic substances of algae and bio toxins of pathogenic microorganisms often introduce from untreated infectious solid waste and liquid effluents. In this way intoxication and bioaccumulation occurs. In this state seafood was found under impacted and contaminated due to localized eutrophication of this coastal area and said to be unsafe for human health when consumed.

Although the coastal area of Karachi Port Trust also receives high concentration of nutrients in the form of reactive nitrogen, it was estimated that average ammonia, nitrogen level in a number of two seawater samples collected from this zone was, i.e., 25.2 ppm. The condition of high TDS and TSS (i.e., 53519 mg/L., & 869.5 mg/L., respectively) of this coastal zone also move towards the direction of

**Table 1.** Phase-1, Sampling regions and Climate-Karachi coastal belt Pakistan

| 1st Phase, October, 2015 | | | | | | |
|---|---|---|---|---|---|---|
| **Karachi coastal regions** | | **Sample description** | | **Field climate conditions** | | |
| **S. No** | **Sampling Locations** | **TP** | **DT** | **TR** | **HT** | **WS** |
| **1** | **PMBQ-A** | SW-Surface | 3m | 36C Sunny | 34% | 10 km/h |
| **2** | **IH-B** | SW-Surface | 10cm | 36C Sunny | 34% | 9 km/h |
| **3** | **SC-FD-C** | SW-Surface | 20cm | 34C Sunny | 34% | 13 km/h |
| **4** | **KPT-FD-D** | SW-Surface | 5m | 33.3C Sunny | 34% | 12 km/h |
| **5** | **HB-FD-E** | SW-Surface | 15cm | 31.5C Sunny | 34% | 10 km/h |

**Locations of seawater sampling: PMBQ, A;** Port Muhammad Bin Qasim., **IH-FD, B;** Ibrahim Haideri., **SC, C;** Sea view-Clifton., **KPT, D;** Karachi Port Trust., **HB, E;** Hawke's Bay
**Field sampling detail: TP;** Type of Sample, i.e. deep or surface., **SW;** Sea Water., **DT;** Sample Depth= It is the description about depth of each specific sample collected from a specific coastal region it is Measured in Meter (m) and centimeter (cm)., **TR;** Day Temperature during sampling was measured in Celsius scale (˚C).
**HT;** Humidity measured in percent (%)., **WS;** Wind Speed measured in kilometer per hour (km/h).

**Table 2.** Phase-2, Sampling regions and Climate-Karachi coastal belt Pakistan.

| 2nd Phase, November, 2015 | | | | | | |
|---|---|---|---|---|---|---|
| **Karachi coastal regions** | | **Sample description** | | | **Field climate conditions** | |
| **S. No** | **Sampling Locations** | **TP** | **DT** | **TR** | **HU** | **WS** |
| **1** | **PMBQ-A** | SW-Surface | 2m | 21C Sunny | 33% | 16km/h |
| **2** | **IH-B** | SW-Surface | 3m | 21C Sunny | 33% | 16km/h |
| **3** | **SC-C** | SW-Surface | 10cm | 21C Sunny | 33% | 12km/h |
| **4** | **KPT-D** | SW-Surface | 5m | 21C Sunny | 33% | 12km/h |
| **5** | **HB-E** | SW-Surface | 15cm | 21C Sunny | 33% | 12km/h |

**Table 3.** Phase-1, Laboratory investigations of seawater of Karachi coastal regions of Pakistan.

| *1st Phase, October, 2015* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Karachi coastal region*** | | ***Lab. Investigation Results*** | | | | | | | | | |
| **S. No** | ***Sample No.*** | **T** | **CL** | **Tur** | **Cond** | **pH** | **TSS** | **TDS** | **DO** | **TN** | **TP** |
| **1** | ***PMBQ-FD-12*** | 32 | 61 | 3.01 | 1400 | 7.30 | 542 | 35011 | 15.59 | 1.643 | 0.961 |
| **2** | ***IH-FD-13*** | 32.4 | 299 | 18.7 | 1016 | 6.2 | 981 | 60201 | 7.38 | 16.362 | 0.993 |
| **3** | ***SC-FD-14*** | 32.4 | 48 | 1.85 | 1207 | 8.52 | 843 | 55873 | 5.53 | 0.576 | 0.264 |
| **4** | ***KPT-FD-15*** | 30.6 | 153 | 12.8 | 1464 | 7.95 | 978 | 70127 | 5.35 | 30.491 | 1.154 |
| **5** | ***HB-FD-16*** | 30.8 | 123 | 3.93 | 1367 | 8.49 | 210 | 35631 | 7.51 | 0.384 | 0.635 |

Locations of seawater sampling: **PMBQ-FD, 12;** Port Muhammad Bin Qasim., **IH-FD,** 13; Ibrahim Haideri., **SC-FD,** 14; Seaview-Clifton., **KPT-FD,** 15; Karachi Port Trust., **HB-FD**-16; Hawke's Bay

**Abbreviation and Unit: T;** Temperature value measured in Celsius scale (˚C)., **CL;** Color value measured in Platinum Cobalt scale (Pt/Co)., **Tur;** Turbidity value measured in Nephelometric Turbidity Unit **(NTU)., Cond;** Conductivity measured in milligram per liter (mg/L)., **pH;** Power of Hydrogen Ion Concentration measured in moles per liter (moles/L). As in the case **PMBQ-FD-12** 1st phase Sample [H+] = 0.5 x 107 mol/L. mol/L, this is the pH of 7.30., TSS; Total Suspended Solids value measured in milligram per liter (mg/L)., **TDS;** Total Dissolved Solids value measured in milligram per liter (mg/L)., **DO;** Dissolved Oxygen value measured in percent (mg/L)., **TN;** Total Nitrogen value measured in milligram per liter (mg/L)., **TP;** Total Phosphorous value measured in milligram per

**Table 4.** Phase-2, Laboratory investigations of seawater of Karachi coastal regions-Pakistan

| 2nd Phase, November, 2015 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Karachi coastal regions** | | **Lab. Investigation Results** | | | | | | | | | |
| **S. No** | **Sample No.** | **T** | **CL** | **Tur** | **Cond** | **pH** | **TSS** | **TDS** | **DO** | **TN** | **TP** |
| **1** | **PMBQ-FD-12** | 28 | 58 | 16.7 | 1149 | 6.9 | 560 | 26260 | 8.77 | 0.8 | 0.95 |
| **2** | **IH-FD-13** | 30.9 | 191 | 61.8 | 1572 | 6.3 | 731 | 39155 | 6.04 | 23.1 | 1.43 |
| **3** | **SC-FD-14** | 27.4 | 144 | 69.8 | 1307 | 7 | 420 | 35070 | 7.78 | 1.6 | 0.2 |
| **4** | **KPT-FD-15** | 27.5 | 75 | 5.91 | 1803 | 6.7 | 761 | 36911 | 8.63 | 20 | 1.08 |
| **5** | ***HB-FD-15*** | 26.9 | 21 | 7.61 | 1549 | 7.3 | 243 | 26800 | 8.53 | 0.2 | 0.1 |

localized eutrophication (Table 2 & 3). However, algal development over seawater of the Ibrahim Haideri coastal area was negatively impacted on the coastal seagrass, as seagrass provides food and shelter to marine fish species and other macro benthos. Although due to blockage of sunlight by thick algal covers over coastal water surface, seagrass and coral reef's growth was found impacted. This was the reason through which it was apparent that the marine fish communities and the other marine slowly yet completely migrated from this coastal zone. The results are tabulated in Table 3 and Table 4.

However, other existing marine life was also under impact due to the extreme environmental stress like deoxygenation of coastal water due to elevated biogeochemical processes often result of eutrophication. In this state mortality rate of coastal life increases (average dissolved oxygen level in a number of two seawater samples at Ibrahim Haideri and coastal areas of Karachi Port Trust-Kemari estimated to be 6.71 mg/L., & 6.99 mg/L).

In this way, adverse environmental conditions of this coastal zone of Ibrahim Haideri and coastal areas of Karachi Port Trust-Kemari were very similar. Marine ecosystem of this coastal region was also found to be suffering from same environmental conditions and, thus, causing a great loss of marine life.

## 4. CONCLUSION

It is concluded from the survey and laboratory investigations of seawater samples that Ibrahim Haideri coastal area and coastal area of Karachi Port Trust-Kemari has witnessed a negative impact owing to the localized eutrophication. The result is in contrast to the results obtained for regions like Port Muhammad Bin Qasim, Seaview-Clifton and Hawke's Bay Beach. The average ammonia, nitrogen level, TDS and TSS in number of two seawater samples of Port Muhammad Bin Qasim, Seaview-Clifton and Hawke's Bay beach were also estimated. However, as compared to Port Muhammad Bin Qasim, Seaview-Clifton and Hawke's Bay coastal zones, sewage effluent, solid waste disposal and industrial effluent runoff through Lyari river and Malir river was potentially impacted on normal ecology and coastal communities of fish and was found the main reason of fish mortalities and seafood contamination in these coastal areas like Ibrahim Haideri and areas of Karachi Port Trust up to Manora channel. One of the most common anthropogenic disturbances of marine ecosystems in the Arabian Sea in Karachi coastline Pakistan was observed. These were found main causes of eutrophication, this coastal pollution and contamination like eutrophication not only impacted on coastal ecology, but also impacted on coastal water quality and recreational facilities of these coastal zones of Sindh-Pakistan.

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Research Article

# Catalase Activity as a Bio-Indicator of Lead+Nickel Toxicity in Carnivorous Fish, *Channa striata*

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**Abstract:** The freshwater ecosystems are extensively polluted with heavy metals discharged from industrial, domestic, and other human activities. These metals are significant stimulators of oxidative stress in aquatic organisms, especially fish, leading to the creation of reactive oxygen species. Therefore, current research was conducted to elucidate the toxic effect of heavy metals mixture (Pb+Ni) on catalase activity in various organs (gills, liver, kidney, brain, muscle and heart) of carnivorous fish, *Channa striata* exposed to sub-lethal concentrations (1/3rd, 1/4th and 1/5th of $LC_{50}$) for a period of 14-day. Fish were sampled for enzyme study after 7 and 14-days. Results showed that all concentrations of metals mixture significantly decreased the CAT activity in selected organs of the fish however, maximum depletion was observed in 1/3rd of $LC_{50}$ concentration followed by the order of 1/4th>1/5th. The CAT activity was decreased with increasing the concentration and duration of exposure.

**Keywords:** Carnivorous fish, Chronic exposure, Metals mixture, Antioxidant enzyme, *Channa striata*

## 1. INTRODUCTION

Contamination of freshwater bodies with a variety of pollutants is a major worldwide issue [1-2]. These pollutants have harmful effects on the aquatic organisms [3]. Among these pollutants, heavy metals are more toxic due to the tendency of bio-accumulation in aquatic ecosystems [4]. Metals present naturally in water bodies or due to the consequences of human activities, have been proven to be a significant factor of exposure to the organism's lives in water, especially fish [5]. These pollutants have harmful effects on fish and are considered to be the most suitable creature for evaluating the aquatic pollution [6].

Normally, nickel (Ni) occurs in traces in individuals, but at higher amount it would be risky to individuals residing in water [7]. Widespread use of nickel in electroplating, ceramic and steel industries which are producing Ni containing products, are releasing untreated waste into water bodies of the Punjab province [8]. Nickel voluntarily makes compound with many ligands upon release into the environment and becomes more mobile as compared to other metals. Nickel may lead to severe problems such as induction of toxicity in organs, contact dermatitis and nickel allergy [9] and also causes morphological transformations in numerous cellular systems and chromosomal aberrations [10].

According to Sfakianakis et al [11] lead is a highly stable heavy metal and is known as a toxic element. Aquatic animals such as fish accumulate lead from water [12]and deposit in different tissues (liver, spleen, gills, kidney and digestive tract)[13]. Lead inhibits the antioxidant enzymes activities, especially thiol-containing antioxidants, and can also stimulate the production of reactive oxygen species (ROS), inducing ''oxidative stress'' [14]. Organisms have antioxidant enzyme system to counteract the oxidative stress. This system includes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione-S-transferase (GST). Theses enzymes also protect the organism against oxy-radical damage such as lipid peroxidation, oxidation of protein and nucleic acids [15].

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Catalase plays important role to minimize the oxidative stress [16-17] by converting hydrogen peroxide into oxygen and water, and facilitate the redox regulation in various tissues [18]. Antioxidants enzymes can be used as a potential biomarker for detection of metal toxicity in freshwater bodies [19] because of their high sensitivity to metals as their activities are significantly altered and prove to be useful in environmental monitoring program [20]. Many studies were conducted to assess the impact of individual metal on fish whereas aquatic animals are commonly exposed to metals mixtures [21]. Therefore, the aim of present research work was to assess the activity of catalase in various organs of carnivorous fish, *Channa striata* under sub-lethal exposure to Pb + Ni mixture.

## 2. MATERIALS AND METHODS

### 2.1. Experimental Lay-out

Freshwater fish, *C. striata* was selected for this experiment. Fingerlings of *C. striata* (90 days old; Average weight, 8.15±0.21) were collected from natural breeding grounds and shifted to the Fisheries Research Farm, University of Agriculture, Faisalabad, Pakistan. Prior to experimental trail, *C. striata* were placed in cemented tanks to acclimatize with laboratory conditions for 14-day. After that, fish were moved to 100-L glass aquarium. A group of fish (n=10) were kept in each aquarium. Control fish were kept in metals mixture free water.

### 2.3. Preparation of Metal Solution

Chemically pure chloride compounds of metals, lead and nickel were dissolved, separately, in deionized water and stock solutions were prepared for required metals and their mixture concentrations (1:1 ratio) on metallic ion equivalence basis.

### 2.4. Metals Mixture Concentration

The 96-h $LC_{50}$ of lead+nickel mixture for *C. striata* was calculated as 52.147 mg $L^{-1}$ by Anum et al [22]. The sub-lethal values for *C. striata* were about 17.382 ($1/3^{rd}$), 13.036 ($1/4^{th}$) and 10.429 ($1/5^{th}$) $mgL^{-1}$. Fish, were exposed to sub lethal concentrations *viz.* 1/3, 1/4, 1/5 of $LC_{50}$ for 14-day. Fish sampling was done after 7 and 14 days.

### 2.5. Water Quality Characteristics

During the experimental period, temperature, total hardness and pH of water was kept constant as 28°C, 230 mg $L^{-1}$ and 7.00, respectively. However, other water variables like magnesium, total ammonia, calcium, sodium, potassium, electrical conductivity and carbon dioxide were also calculated and maintained [23].

### 2.6. Preparation of Organ Homogenate

To isolate catalase, fish tissues viz. liver, gills, kidney, brain, heart and muscle of *C. striata* were separated. All the organs were weighed and homogenized in phosphate buffer (pH 7.0) in ratio of 1:4 (w/v) for 15 minutes by using homogenizer with short intermissions. The muslin cloth was used to eliminate the debris from homogenized tissues. The obtained filtrate was centrifuged in refrigerated centrifugal machine for 15 minutes at 10,000 rpm and 4°C. Supernatant was separated for enzyme analysis.

### 2.7. CAT Activity

Catalase activity was measured by its ability to reduce the $H_2O_2$ concentration at 240 nm [24]. In a cuvette 2 mL of blank solution (60 mM Phosphate buffer used as blank) was taken and put into the spectrophotometer and it was set to zero at wavelength of 240 nm. Buffer substrate solution of 10 mM of $H_2O_2$ was prepared in 60 mM phosphate buffer. In a cuvette containing buffered substrate solution (1.95 mL), enzyme extract (0.05 mL) was added and placed into the spectrophotometer. The reaction time was 3 minutes and the absorbance was checked after interval of 3 minute.

### 2.8. Statistical Analyses

Data obtained from this study were statistically analyzed by using Statistix 8.1 and significant difference between treatment groups was tested by one-way analysis of variance (ANOVA).

## 3. RESULTS

The catalase activity was significantly depleted in all selected organs *viz.* liver, gills, kidney, brain, muscle and heart of exposed fish in comparison of control. This indicates a reduced activity to protect the cells against $H_2O_2$. Results showed that all concentrations of metals mixture were significantly decreased the CAT activity however; maximum depletion was observed in $1/3^{rd}$ of $LC_{50}$

concentration followed by that of 1/4th and 1/5th. The CAT activity decreased with increasing the concentration and duration of exposure. Graphical representation of data is given in Fig. 1-6.

## 4. DISCUSSION

Metals contribute in oxidative stress by producing reactive oxygen species in two ways. Redox active metals (vanadium, iron, copper and chromium) produce ROS via redox cycling. Redox inactive metals (nickel, mercury, lead, and cadmium) have potential to disrupt antioxidants and enzymes which are thiol-containing [25-26]. Fenton reaction is a third important phenomenon of ROS production in which hydrogen peroxide oxidizes the ferrous iron (II) to ferric iron (III), a hydroxyl anion, and a hydroxyl radical [27]. The superoxide radical can reduce iron to its ferrous form. Heavy metals which are involved in Fenton reaction include chromium, titanium, copper, cobalt, vanadium, and their complexes [28]. Antioxidant enzymes are vital to neutralize the oxidative stress induced by heavy metals once the supply of other antioxidant compounds is reduced [29].

Fish body organs are gifted with defensive mechanism [30] which includes antioxidant enzymes (i.e. superoxide dismutase, catalase, glutathione peroxidase, glutathione S transferase and glutathione reductase) to save them from oxidative stress. Catalase is primarily present in peroxisomes and trapped hydrogen peroxide ($H_2O_2$) [31]. Catalase transforms the hydrogen peroxide

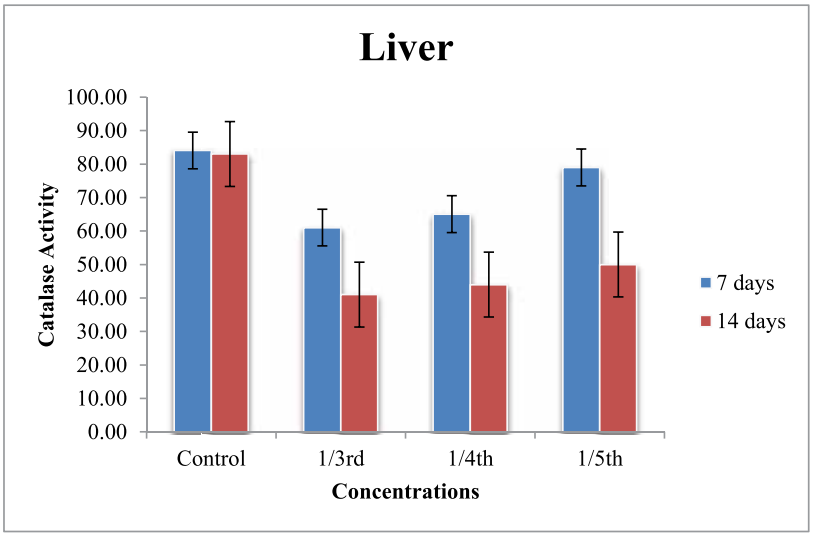


**Fig. 1.** Activity of CAT (U/mL) in liver of *C. striata* exposed to metals mixture for different time intervals

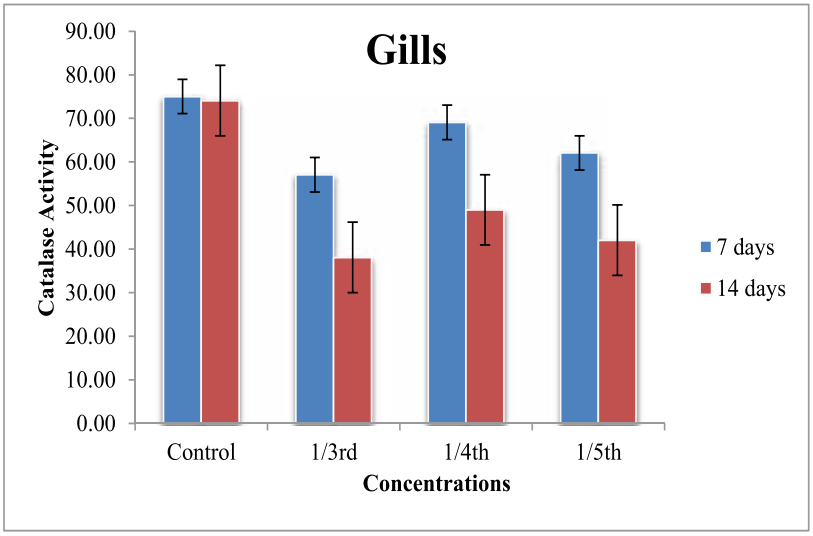


**Fig. 2.** Activity of CAT (U/mL) in gills of *C. striata* exposed to metals mixture for different time intervals

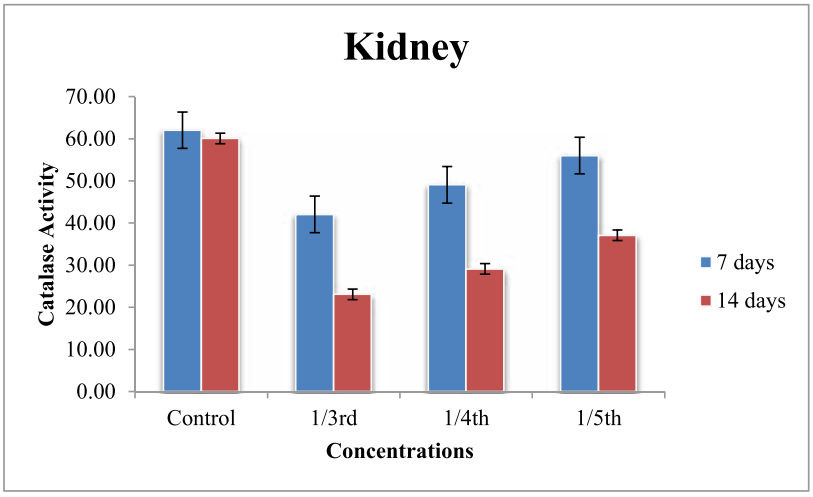


**Fig. 3.** Activity of CAT (U/mL) in kidney of *C. striata* exposed to metals mixture for different time intervals

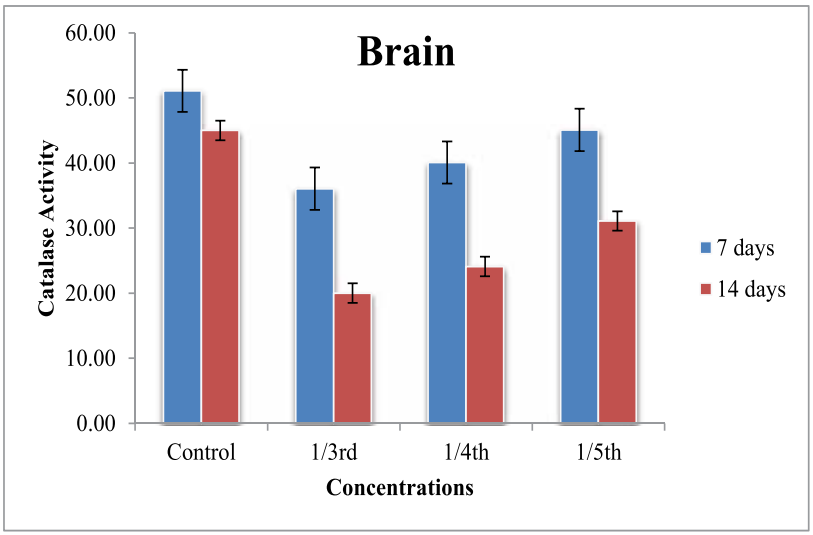


**Fig. 4.** Activity of CAT (U/mL) in brain of *C. striata* exposed to metals mixture for different time intervals

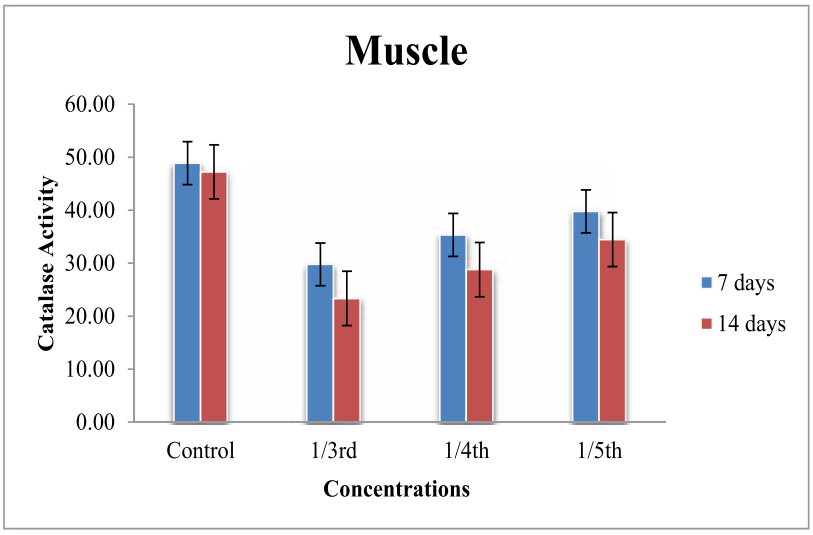


**Fig. 5.** Activity of CAT (U/mL) in muscle of *C. striata* exposed to metals mixture for different time intervals

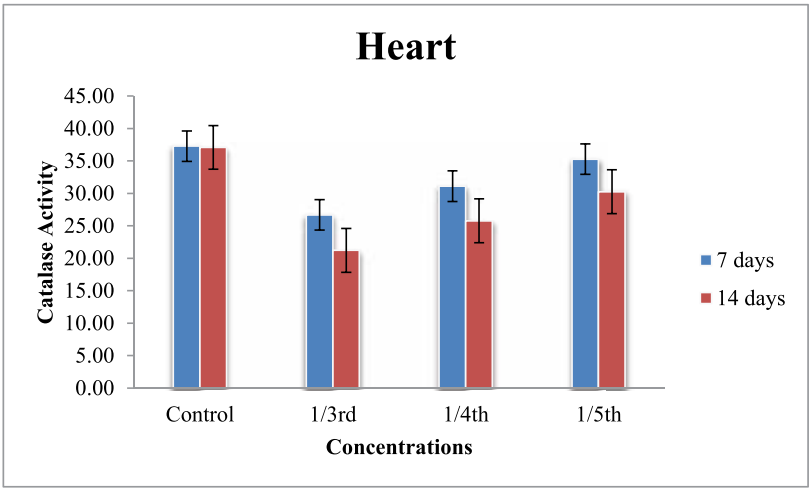


**Fig. 6.** Activity of CAT (U/mL) in heart of *C. striata* exposed to metals mixture for different time intervals

into water and oxygen, to protect the cell from the accumulation of $H_2O_2$. This indicates that the decreased activity may be due to protection of cell against $H_2O_2$.

Many authors have studied the effect of heavy metals on antioxidant systems of herbivorous and omnivorous fish. However, very little information is present in literature in which metals mixture effect was studied on carnivorous fish antioxidant systems. Our results showed that metallic ion concentration had negative impact on activity of catalase. CAT activity was depleted in metals mixture stressed fish in comparison of control. These finding are in accordance to Farombi et al [32]who observed the lower level of CAT in hepatic, gills, cardiac and renal tissues of African catfish exposed to heavy metals(cadmium and copper). The response of CAT activity may vary with the environmental factors, duration of exposure and type of toxicants [20].

Fish increased/decreased the level of antioxidants to overcome the oxidative stress [33]. According to Madhavan and Elumalai [34] the activity of CAT decreased in the gill and kidney of fish, *Clarias batrachus* under chromium exposure. Velma and Tchounwou [35] observed decreased CAT activity with increasing the concentration of chromium. According to Shen et al [36], CAT activity decreased with increasing the exposure concentration and duration. Depleted CAT activity in liver, gills and kidney of goldfish under chromium exposure was recorded by Kubrak et al [37].The activity of CAT decreased in chromium exposed fish [38]. Sub-lethal exposure of cadmium chloride significantly inhibited the CAT activity in gills, muscle, heart and liver of *Oreochromis niloticus* [39]. According to Saliu and Bawa-Allah [40] CAT activity decreased in hepatic tissues of *Clarias gariepinus* after sub-lethal exposure to lead and zinc. Yilmaz et al [41] reported the lower CAT activity in *Cyprinus carpio* in comparison to uncontaminated area. Exposure of cadmium significantly inhibited the CAT activity in renal tissues of the sea bass. This reduction in activity may be due to the direct binding of cadmium to CAT [42]. Similar result was observed by Sunaina and Ansari [43] who reported the reduced CAT activity in liver of zebra fish exposed to cadmium.

## 5. CONCLUSION

The findings of current study revealed that chronic exposure of heavy metals mixture can cause an imbalance in the antioxidant enzymes activities such as catalase in fish. Furthermore, it was concluded that these enzymes could be successfully used as prospective biomarkers of heavy metal toxicity to the freshwater fish in aquatic environment.

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Research Article

# Antibacterial Activity and Composition of Crude Extracts of Kaffir Lime (*Citrus hystrix* DC.) Leaves and Callus

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**Abstract:** Kaffir lime extracts contain bioactive compounds that have anti-bacterial properties. However, the production of bioactive compounds varies throughout the year, which limits its use as an antibacterial agent. Therefore, we have used callus induction, an in vitro culture technique, to provide controlled conditions for studying the activity of kaffir lime extracts from kaffir lime leaves and callus. Callus was induced from seed explants. Extraction of bioactive compound was performed by the maceration method using ethyl acetate or chloroform. Bacterial inhibition zone was determined using the Kirby-Baueur method with modifications. Analysis of bioactive compound was done using GC-MS. The results showed that all extracts had antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The most potent extracts against *S. aureus* and *E. coli* were generated using chloroform and ethyl acetate respectively. Furthermore, the antibacterial mechanism of kaffir lime leaves extracts was determined to be bacteriostatic. Both chloroform and ethyl acetate extracts had several bioactive compounds that inhibit bacterial growth. In addition, kaffir lime callus extracts inhibit *S. aureus* and *E. coli.* Together, these data reveal that kaffir lime callus also has broad spectrum antibacterial activity. Although the antibacterial activity of callus was less than that of leaves, these results warrant the further investigation of kaffir lime's callus to produce antibacterial agents.

**Keywords:** Anti-bacteria, callus, *Escherichia coli*, kaffir lime, leaves, *Staphylococcus aureus*

## 1. INTRODUCTION

The use of antibiotics to treat bacterial infection is associated with hypersensitivity, super-infection and organ toxicity [1]. Therefore, additional research is required to develop antibacterial agents that have fewer side effects on normal cells [2]. Natural products contain secondary metabolites which can be used as an antibacterial agent. Furthermore, secondary metabolites also perform an additional function of immunostimulant, so the consumption of a natural product can act directly to kill infectious agents and indirectly by increase the activity of immune system to eliminate those infectious agents [3].

Kaffir lime (*Citrus hystrix*), is a variety of citrus which is native to Indonesia, Malaysia and Thailand. Kaffir lime contains many secondary metabolites which can be used as an antibacterial agent such as flavonoids, alfa-tocopherol, limonoids, alkaloids, glycerophospholipids, terpenoids etc. [4, 5]. In general, production of secondary metabolites is affected by external factors such as biotic and abiotic environment [3]. Unfortunately, the content of bioactive compounds in natural medicines is unstable throughout the year which affects their use as a reliable method to treat bacterial infections [6]. Thus we need biotechnical approaches such as tissue culture to maintain the quality and even increase the quantity of secondary metabolites. The use of tissue culture for secondary metabolites production can be used as alternative method since we can control environmental factors [7].

This study examines the effects of kaffir lime extracts on both gram positive and gram negative bacteria. *Staphylococcus aureus* is gram positive bacteria which can cause dermatitis, acne pustule, nosocomial infection and toxic shock syndrome,

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while *Escherichia coli* is gram negative bacteria which can cause diarrhoea, urinary tract infection and meningitis/sepsis. Natural products are categorized as having antibacterial activity if MIC (minimum inhibitory concentration) value < 1 mg $mL^{-1}$ [8]. Therefore the objective of this study was to analyze the antibacterial activity of kaffir lime leaves and callus against *S. aureus* and *E. coli* and to determine the MIC value associated with their antibacterial properties.

## 2. MATERIALS AND METHODS

### 2.1 Preparation Simplicia Powder and Extraction

Samples of leaves were taken in Magelang, Central Java, Indonesia. The samples were then dried until a constant dry weight was obtained and made into a fine powdered kaffir lime leaves. Leaves powder was extracted by maceration method using methanol, ethyl acetate and chloroform to get paste extract. Then, the serial concentration of extract solution has been made by 100 % DMSO (dimethyl sulfoxide).

### 2.2 Induction and Cultivation of Kaffir Lime Callus

Callus induction was done according to induction method of callus Citrus rootstock with some modifications [9]. Seeds of kaffir lime were sterilised in 5.25 % Clorox for 3 min and rinsed twice with sterilised distilled water in Laminar Air Flow (LAF). Seeds were cut in half and induced on MS standard (Murashige and Skoog) medium which contain 8 g $L^{-1}$ agar and 30 g $L^{-1}$ sucrose. pH of the medium was adjusted in 6 by adding 1 N HCl and 1 N KOH. MS medium supplemented with growth regulator 2.4-D and BAP 1:0.5 mg $L^{-1}$ was used to callus induction. Callus was taken after 40 days of incubation.

Callus was dried and then grounded into powder with mortar and pestle. Callus powder was extracted by maceration method. Firstly, we use methanol, ethyl acetate and chloroform extracts of leaves then empirical determinations were made to determine the best solvent for each bacteria. According to leaves experiment, callus was extracted using the best solvent for each bacteria. Ethyl acetate extract was used to test *E. coli* while chloroform extract was used for *S. aureus* experiment. The sample was put in solvent for 24 h. After that, sample solution was filtered through Whatman paper.

### 2.3 Medium and Samples Preparation

Antibacterial testing was conducted using Mueller Hinton Agar (MHA) which contained 0.2 % beef extract, 1.75 % acid hydrolysate of casein, 0.15 % starch and 1.7 % agar with final pH 7.3. Purification of bacteria was done to get 24 h bacterial culture. Then colony was taken and suspended in NaCl 0.85 % solution. McFarland 0.5 ($1.5 \times 10^8$) was used for standardisation of some colonies [10].

### 2.4 Measurement of Inhibition Zone

Methanol, chloroform and ethyl acetate extracts were dissolved in DMSO 100 %. Well difussion method was used and was modified from Kirby-Baueur method according to NCCLS (*The National Committe for Clinical Laboratory Standard*) [10]. Tetracyclin 1 mg $mL^{-1}$ was used as a reference. Clear area around extract was determined as inhibition zone. The best extract was determined based on the widest clear zone result among those extracts. Only the best extract was used for further experiments.

### 2.5 Growth Curve of Bacteria

The agar dilution method was employed to count the bacterial growth after extract exposure. Counting of the colony was made in 24 h, 48 h and 72 h after incubation. Then, the growth curve was made to determine whether the action mechanism characteristics of those extracts are bactericidal or bacteriostatic.

### 2.6 Analysis of Bioactive Compounds

Kaffir lime leaves and callus extracts were then analysed by GC-MS Shimadzu GCMS-QP 2010S, with a non-polar column AGILENT HP-5 MS with 95 % dimethylpolysiloxane and 5 % diphenyl polysiloxane to determine the profile of leaves bioactive compounds.

### 2.7 Data analysis

Data analysis was done using ANOVA ONE WAY

SPSS 16 and regression analysis.

## 3. RESULTS AND DISCUSSION

Kaffir lime (*Citrus hystrix*), is a variety of citrus which is native to Indonesia, Malaysia and Thailand. The taxonomical classification of *Citrus hystrix* DC. is

| | |
|---|---|
| Kingdom | : Plantae |
| Division | : Spermatophyta |
| Sub Division | : Angiospermae |
| Class | : Dicotyledonae |
| Order | : Rutales |
| Family | : Rutaceae |
| Genus | : Citrus |
| Spesies | : *Citrus hystrix* DC [11] |

### 3.1 Antibacterial Activity of Kaffir Lime Leaves

Inhibition zone of all of those extracts against *S. aureus* was shown in this Fig. 1 and Fig. 2. The result showed that all extracts have antibacterial activity against *S. aureus*. However, chloroform extract showed the best inhibition activities compared to other solvents.

Growth curve of *S. aureus* showed that inhibition mechanism of chloroform extract was bacteriostatic Fig. 3. Inhibition zone of all extracts against *E.coli* is shown in Fig. 4 and Fig. 5.

The result showed that all extracts have antibacterial activity against *E. coli* However,

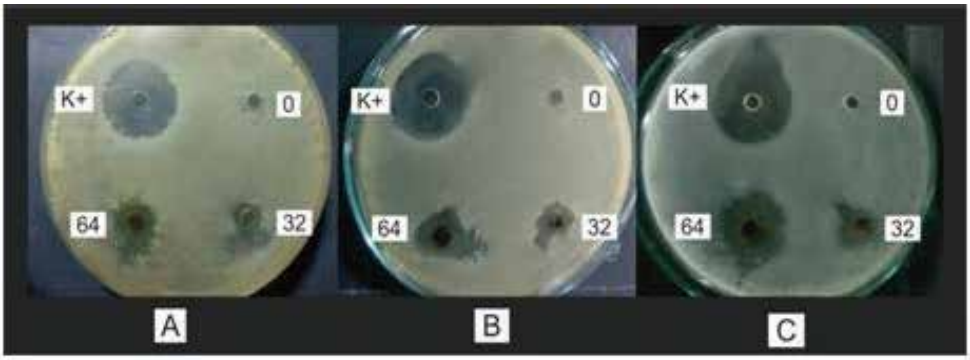


**Fig. 1.** Inhibition zone of methanol (A), ethyl acetate (B) and chloroform (C) leaves extract against *S. aureus* FNCC 0047

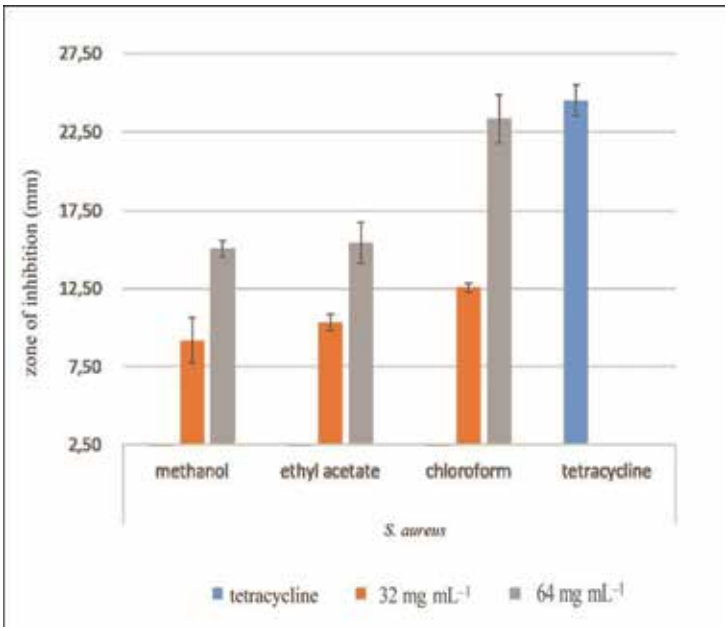


**Fig. 2.** Diameter of zone of inhibition of Kaffir Lime leaves extracts against *S. aureus* FNCC 0047

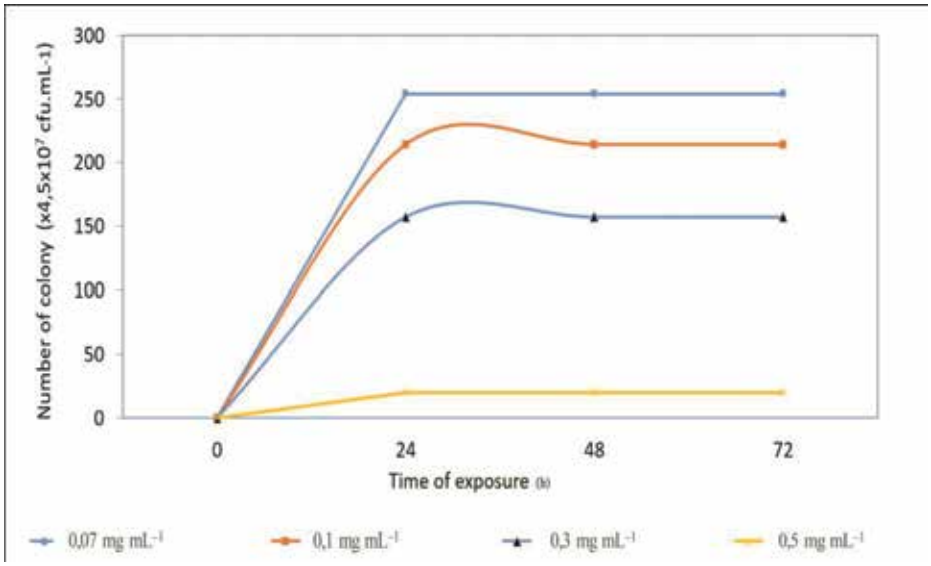


**Fig. 3.** Growth curve of *S. aureus* after treated with several concentrations of chloroform extract of kaffir lime leaves after 24 h, 48 h, and 72 h incubation

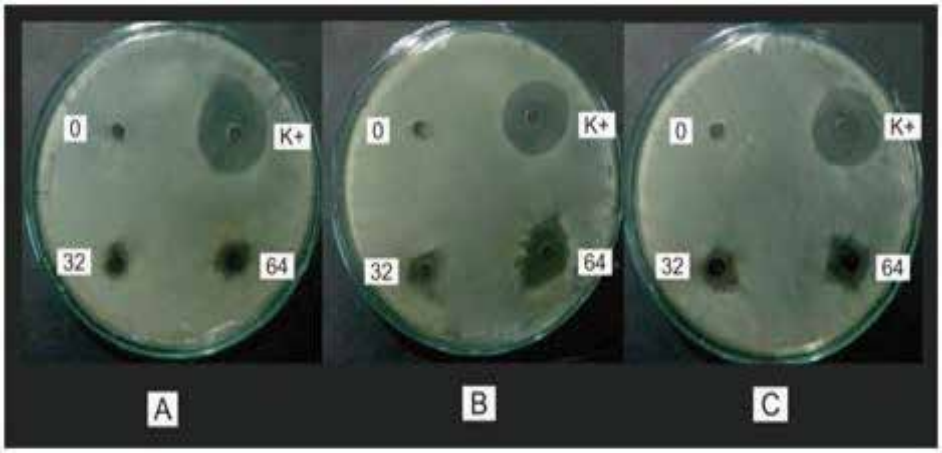


**Fig. 4.** Inhibition zone of methanol (A), ethyl acetate (B) and chloroform (C) leaves extract against *E. coli* FNCC 0091

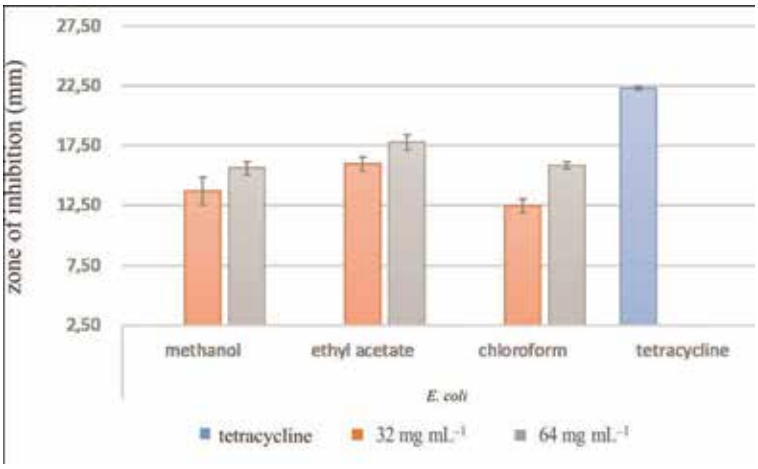


**Fig. 5.** Diameter of zone of inhibition of kaffir lime leaves extracts against *E. coli* FNCC 0047

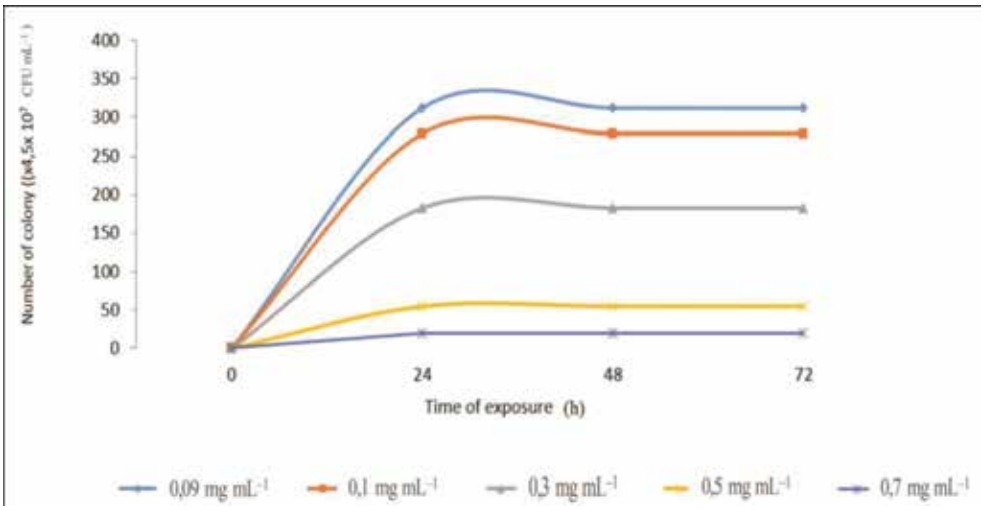


**Fig. 6.** Growth curve of *E. coli* after treated with several concentrations of ethyl acetate extract of kaffir lime leaves after 24 h, 48 h, 72 h of incubation

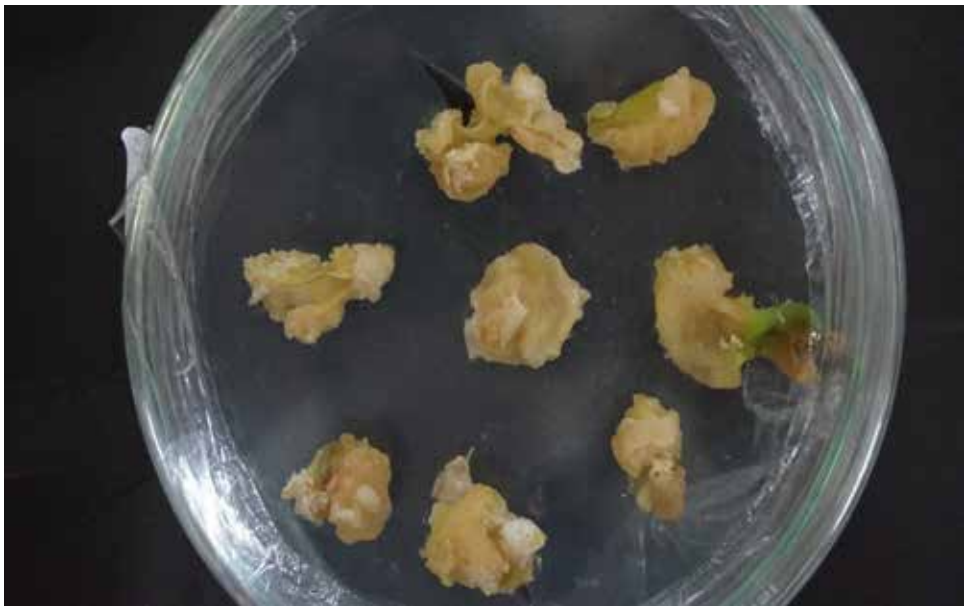

**Fig. 7.** Kaffir lime callus from seed explant

ethyl acetate extract had the best inhibition activity compared to other solvents.

Diameter of inhibition zone result can be used to categorize antibacterial activity. Natural products are categorised to be strongly active if the diameter zone is more than 18 mm, active if diameter zone is in the range of 13 mm to 18 mm, less active if diameter zone is in the range of 9 mm to 12 mm and inactive if diameter zone is less than 9 mm [8]. Ethyl acetate extract with concentration of 64 mg $mL^{-1}$ produced inhibition zone (17.75 ± 0.66) mm against *E. coli*. Hence, ethyl acetate extract was categorised as active against *E. coli*. On the other hand, the same concentration of chloroform extract has produced inhibition zone (23.33 ± 1.52) mm against *S. aureus*. Therefore, chloroform extract had strong activity against *S. aureus*. This phenomenon showed inhibition in a dose-dependent manner, meaning that the activity of those extracts can be enhanced by increasing their concentration.

### 3.2 Antibacterial Activity of Kaffir Lime Callus

Kaffir lime callus can be produced by inducing seed in MS medium supplemented with growth regulator 2.4-D and BAP. The 2.4-D was synthetic auxin which can be used as herbicide and it can trigger cell to proliferate because of stress responses, while BAP is synthetic cytokine which has main function

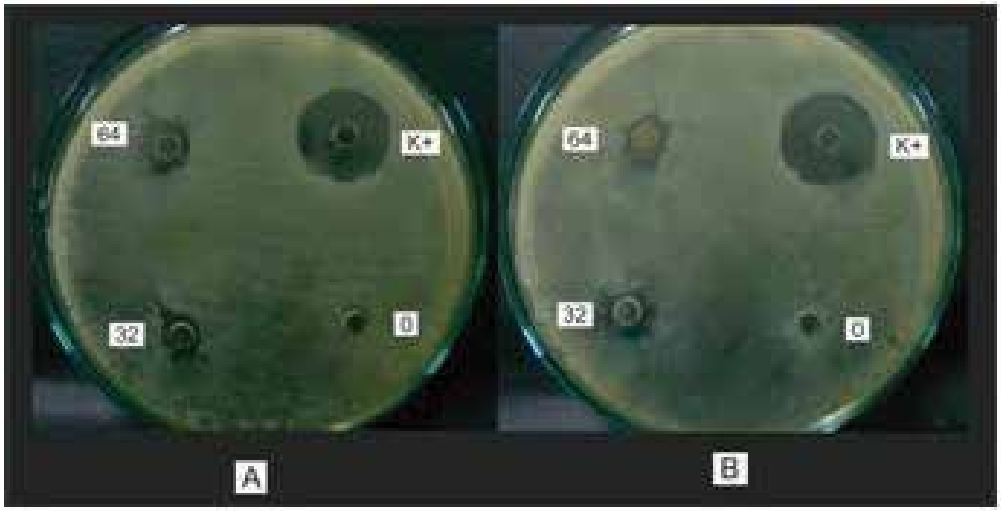


**Fig. 8.** Inhibition zone of kaffir lime callus ethyl acetate extract against *E. coli* and chloroform extract against *S. aureus* (concentration: 0 mg mL$^{-1}$, 32 mg mL$^{-1}$, 64 mg mL$^{-1}$) and tetracycline 1 mg mL$^{-1}$ as reference.

**Table 1.** Inhibition zone of callus ethyl acetate extract against *E. coli*

| Concentration (mg mL$^{-1}$) | Zone of inhibition (mm) |
|---|---|
| 64 | 13.75 ± 0.43$^{b}$ |
| 32 | 13.5 ± 1.25$^{a}$ |

* Note: Inhibition zone was including diameter of well (5 mm). Different letter showed significant difference

**Table 2.** Inhibition zone callus chloroform extract against *S. aureus.*

| Concentration (mg mL$^{-1}$) | Zone of inhibition (mm) |
|---|---|
| 64 | 12.08 ± 0.94$^{b}$ |
| 32 | 11.58 ± 0.28$^{a}$ |

* Note: Inhibition zone was including diameter of well (5 mm). Different letter showed significant difference

in cell division.

Both callus extracts can be categorised as antibacterial activity. Based on the measurement of inhibition zone, the activity of callus extract can be categorised into several groups. At the concentration of 64 mg mL$^{-1}$ (6.4 %), ethyl acetate extract of callus can be categorized to be active against *E. coli*, while the chloroform extract was less active against *S. aureus*. This phenomenon showed a dose-dependent relationship and suggested that the activity can be increased by adding more concentration of extract into bacterial culture.

The antibacterial activity of callus was less than than of leaves. Therefore it is important to do elicitation to callus to increase the content of secondary metabolite possessing antibacterial activity.

### 3.3 Bioactive Compounds of Kaffir Lime Leaves and Callus that Have Antibacterial Activity

Chloroform and ethyl acetate extracts of kaffir lime leaves were analyzed with GC-MS to determine bioactive compounds in kaffir lime leaves. In ethyl acetate extracts, there were found 52 bioactive compounds namely β-citronellol, phytol; citronellyl acetate; palmitic acid; linolenic acid; citronellyl propionate; nerolidol 2; α-farnesene; 1-methyl cyclohexene; citronellal; caryophyllene; delta-cadinene; hexacosane; eicosane; vitamin E; 1, 2, 3-propanetriol; monoacetate; 4-methyl-1,

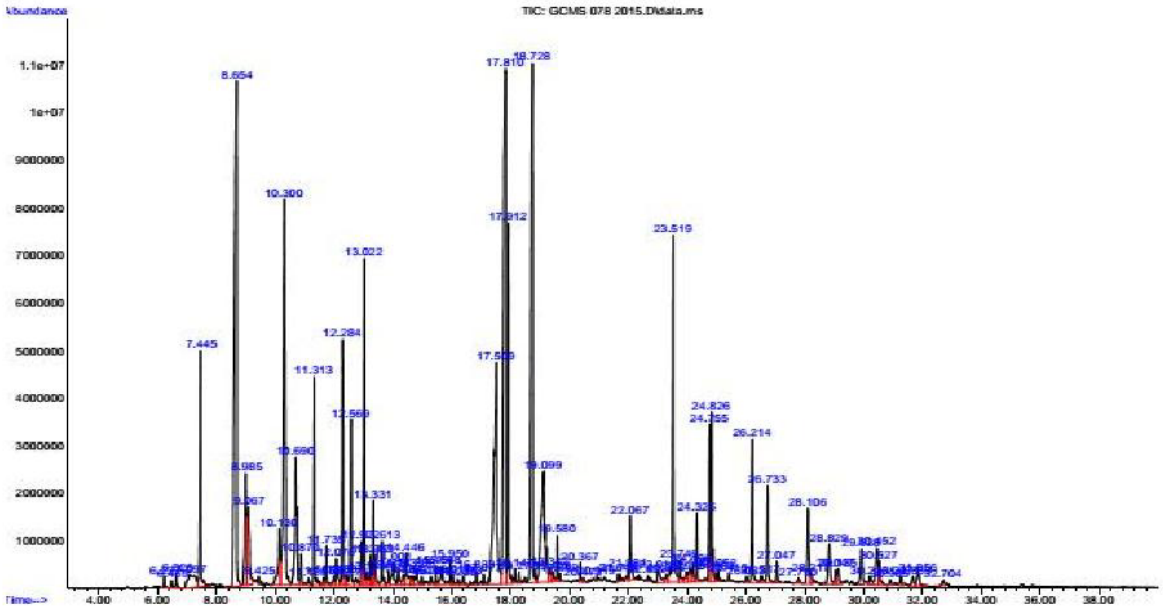

**Fig. 9.** GC-MS Chromatogram of ethyl acetate extract of *Cytrus hystrix* leaves

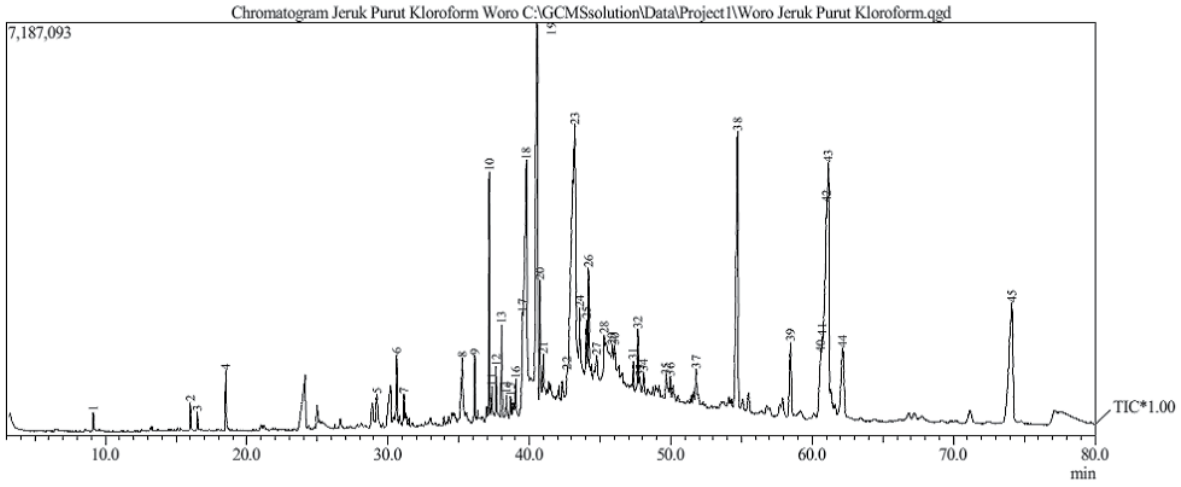


**Fig. 10.** GC-MS Chromatogram of chloroform extract of *Cytrus hystrix* leaves

4-heptadiene; γ-sitosterol; spathuleno; lupeol; squalene; torreyol/ cedreanol, germacrene; elemol; cetene; caryophyllene oxide; trans-farnesol; phytol acetate; hydroxyfuranocoumarin; α-caryophyllene/ α-humulene; ethyl palmitate; dotriacontane; dihydrolanosterol; neophytadiene; α-terpinolene, 1-octadecene (CAS) ; Z, Z-10, 12-hexadecadien-ol acetate; borane; dimethylmethyl (CAS), napthalene; citronellyl propanoate; 17-pentatriocontane; lupeyl acetate; margaric acid; trans-linalool oxide; dotriacontanol; 13-methyl-tetradec-13-ene-1,12-dio; farnesol; campesterol; benzenne propanoic acid; β-tochopherol and 1, 7-nonadiene, 4, 8, -dimethyl (Figure 9).

In chloroform extracts, 32 bioactive compounds were identified namely 9, 12, 15-octadecatrien-1-ol; 6-octen-1-ol, 3, 7-dimethyl; propionate; palmitic acid/ hexadecanoic acid; 1, 5, 9-decatriene, 2, 3, 5, 8-tetramethyl; heneicosane; neophytadiene; 14b-pregnane; heneicosane; neophytadiene; isophytol; 9-tricosene; citronellyl acetate; tetradecanoic acid/myristic acid; 2, 10-dodecadien-1-ol-3, 7, 11-trimethyl; 9-octadecanoic acid (CAS); phytol; cyclooctacosane; 3-eicosane; citronella; 6-octen-1-ol, phenol, 3, 5-bis(1,1-dimethylethyl); 2-hexadecen-1-ol, 3, 7, 11, 15-tetramethyl; 1-eicosanol; benzene,1-methoxy-2-[(4-methoxypheny) methyl] ; spathulenol;

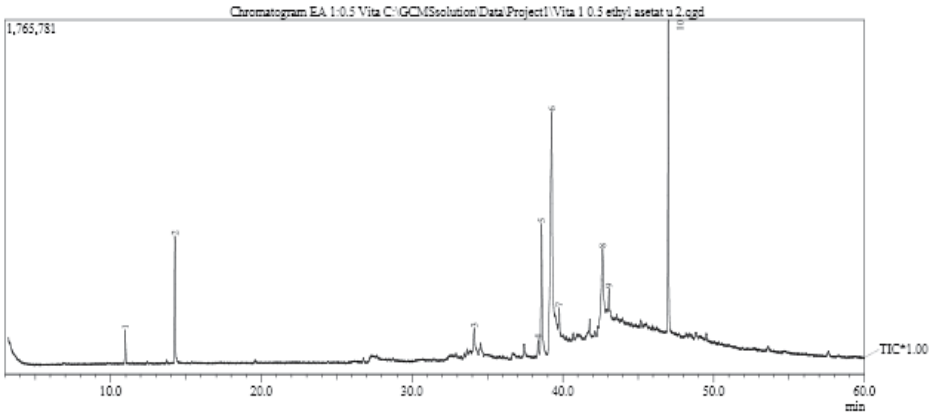


**Fig. 11.** GC-MS Chromatogram of ethyl acetate extract of *Cytrus hystrix* callus

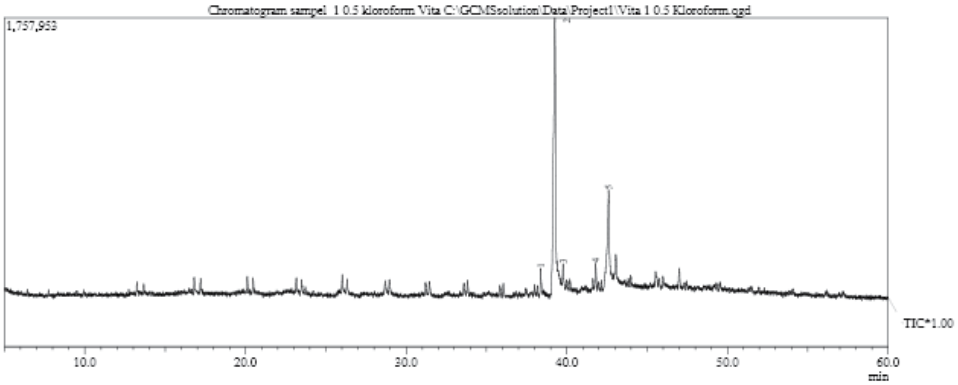


**Fig. 12.** GC-MS Chromatogram of chloroform extract of *Cytrus hystrix* callus

phytol isomer; trans-linaloloxide; citronellyl propionate; hexanedioic acid; 2-hexadecene, 3, 7, 11, 15-tetramethyl; 1, 2-benzenedicarboxylic acid, bis(2-ethylhexyl)ester; styrene; and eicosane (Figure 10).

The type of solvent in extraction can affect the yield, which will also make the determination of the amount of bioactive compounds in an extract difficult. Since bioactive compounds have different chemical characteristics and polarities they can or cannot be soluble in a particular solvent. Hence different solvent with different polarity can affect the efficacy of the extraction. According to the result, *S. aureus* was more sensitive by the exposure to chloroform extract compared to another extract, while the *E. coli* was more sensitive to ethyl acetate extract than another. This indicates that each extract contains a bioactive compound that is only preserved in a particular solvent.

Based on GC-MS analysis of chloroform extract, it was shown that those extracts contained secondary metabolite which was dominated with terpene, followed by fatty acids. According to the recent publication, it was known that chloroform leaves extract contains some potential antibacterial bioactive compounds, such as neophytadiene, squalene, isophytol, citronellyl acetate, myristic acid, 9-octadecanoic acid, phytol, 1-eicosanol, spathulenol, phytol isomer, trans-linalool oxide and eicosane. On the other hand, ethyl acetate extract contains some potential antibacterial agents such as beta-citronellol, phytol, citronellyl acetate, palmitic acid, linolenic acid, citronella, caryophyllene, eicosane, vitamin E, gamma sitosterol, spathulenol, squalane, germacrene, trans-farnesol, hydroxyfuranocoumarin, neophytadiene, alpha-terpinolene, naphthalene, trans-inalooloxide and farnesol. Although antibacterial mechanism for each compound remains unclear, previous studies showed that some secondary metabolites could damage bacterial membrane. This action belonged to farnesol [12].

On the other hand, ethyl acetate callus extract contained palmitic acid, oleic acid, alpha-pinene and 1.8-cineole (Fig. 11). However, any secondary metabolite was not detected in the chloroform extract (Fig. 12). In the chloroform extract hexadecanoic acid, palmitic acid and oleic acid had been detected. These fatty acids have antibacterial activity. These data supported previous data about inhibition zone between leaves and callus. The antibacterial activity of callus was less than that of leaves because secondary metabolite in callus was less than in leaves.

1.8-cineole is known to be anti-*E. coli* while alpha-pinene can act as antibacterial by damaging bacterial membrane. Alpha-pinene and 1.8-cineole belonged to terpenes groups with antibacterial property. The concentration of those compounds can be increased using some methods such as adding some elisitor to give a stress exposure or adding an enzyme precursor after making an analysis in their metabolic pathway [13]. Fatty acid such as palmitic was known to be able to inhibit the growth of bacteria by damaging cytoplasmic membrane [14].

## 4. CONCLUSION

Both leaves and callus extracts of kaffir lime had broad spectrum antibacterial activity which can inhibit the growth of *S. aureus* and *E. coli*. The best solvent against *S. aureus* is chloroform extract while for *E. coli is* ethyl acetate extract. Furthermore all tested extracts are bacteriostatic in their mechanism of action against each bacterial species. Although the antibacterial activity of callus is less than that of leaves, these results warrant further investigations of kaffir lime's callus to produce antibacterial agent.

## 5. ACKNOWLEDGEMENTS

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Research Article

# Differential Performance of Wheat Genotypes for Grain Yield, Phosphorus Uptake and Utilization at Low and High Phosphorus Levels

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**Abstract:** A pot experiment was conducted to test grain yield response and phosphorus use efficiency (PUE) in 10 wheat genotypes at low (10 mg P $kg^{-1}$ soil) and high (40 mg P $kg^{-1}$ soil) soil phosphorus (P) levels. The genotypes differed significantly ($P < 0.05$) for grain yield, P uptake and P efficiency indices at each P level. Phosphorus stress factor (PSF) varied between 0.15 and 30.1%, signifying the differential P responsiveness of the wheat genotypes. Each parameter was assigned an index score of 1, 2, or 3 for its low, medium, or high grade of performance at each P level. Furthermore, the genotypes were grouped into six categories based on their grain yield and total P uptake at low P level. Genotypes EST-28/11 and MSH-3 with high grain yield (8.42 and 7.95 g $pot^{-1}$) and high P uptake (38.0 and 30.9 mg P $pot^{-1}$), and NIA-Sunder with high grain yield (8.33 g $pot^{-1}$) but medium P uptake (23.9 mg P $pot^{-1}$) at low P level can be selected for P deficient soils. Genotypes EST-28/11, MSH-3 and NIA-Sunder also attained high total index scores (25, 23 and 23) at low P level. Such type of categorization will aid in breeding programs for improving nutrient use efficiency.

**Keywords:** Phosphorus efficiency index, Phosphorus utilization, Plant nutrition, Wheat genotype.

## 1. INTRODUCTION

Phosphorus (P) deficiency is a major constraint to crop productivity on more than 30% of the world's cultivated soils [1] and about 5 to 15% of yield losses are attributed to the deficiency of this nutrient [2]. In case of Pakistan, P deficiency has been reported on more than 90% of soils. High pH and calcium carbonate contents impart P fixing properties to the Pakistani soils which entail into low recovery (15-20% only) of applied P fertilizers [3]. Inorganic P fertilizers are manufactured from mined rock phosphate (RP) which is a limited and non-renewable resource, and expected to deplete by the end of this century [4]. Diminishing RP reserves, geographical concentration of RP in only very few countries of the world, low recovery and high prices of P fertilizers, and environmental pollution due to P losses warrant the global stakeholders to come up with multi-dimensional and sustainable strategies to resolve this problem [5]. The development of crop cultivars that uptake and/or utilize P more efficiently on P deficient soils seems a sustainable alternative to the conventional high input approach [6]. Cultivation of P efficient crops will significantly improve the efficiency of P fertilizers and decrease the cost of production as well as environmental deterioration. Genotypic variation for P efficiency has been reported in many crops like rice [7], barley [8], Brassica [9], wheat [6] and cotton [10] which can be exploited to uphold the productivity of P impoverished agricultural systems.

Phosphorus efficiency in any crop can be improved by the screening and categorization of existing germplasm for growth and yield performance in low P medium. Such type of classification will lead to the identification of genotypes that can be effectively grown on soils with variable P contents. Some researchers have classified the crop genotypes based on their biomass production and P utilization [6, 11] and

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identified two categories for each of efficiency and response, i.e. i) efficient and responsive, ii) efficient but non-responsive, iii) inefficient but responsive, and iv) inefficient and non-responsive. However, the usefulness of such classification was challenged by Aziz et al. [12] as no sharp distinction between efficiency as well as response groups could be made. A cultivar may be categorized as efficient or inefficient if it has mean value slightly higher or lower than the population mean. Contrarily, Gill et al. [13] proposed nine different classes of wheat cultivars by employing metroglyph technique with three categories viz., low, medium and high of efficiency and responsiveness each. Such type of classification was very effective and could accommodate a wide range of medium efficient or responsive cultivars between low and high extremes.

Wheat, the staple food of Pakistan, is being sown on more than 9 million hectares annually, with production of about 25 million tons [14]. It contributes to 1.9 % of GDP of the country and 9.6 % of the value addition in agriculture. A major chunk of production cost of wheat crop is associated with P fertilizer inputs. According to NFDC [15], wheat crop accounts for 50% of annual P fertilizer consumption in Pakistan. In this perspective, improvement in P use efficiency of wheat crop alone can significantly affect P fertilizer demand in future. The current study was, therefore, undertaken to evaluate the growth and yield response of 10 wheat genotypes at low and high P levels in soil.

## 2. MATERIALS AND METHODS

Seeds of 10 wheat genotypes were collected from Plant Breeding and Genetics Division, Nuclear Institute of Agriculture (NIA), Tando Jam. Genotypes included nine advanced wheat lines (AA-V1, AA-V2, AA-V3, MSH-3, MSH-5, BWQ-4, EST-28/11, EST-29/9, ESW-9525) and one cultivar (NIA-Sunder). Soil was collected from the experimental field of NIA, Tando Jam. It was air-dried, crushed to pass through a 2 mm sieve and subsequently filled in plastic pots of 10 kg capacity (25 cm diameter and 30 cm depth). The soil used in the pot experiment was clay loam in texture (with 21.7, 42.2 and 36.1% sand, silt and clay, respectively), non-saline ($EC_e$ = 2.1 dS $m^{-1}$), alkaline (pH = 7.9) with 12.5% $CaCO_3$, 0.73% organic matter, 0.081% Kjeldahl N, and 3.7 and 180 mg $kg^{-1}$ AB-DTPA (ammonium bicarbonate - diethylene triamine penta acetic acid) extractable P and K, respectively.

The experiment was laid-out in a completely randomized design with factorial combination of 10 wheat genotypes and two phosphorus levels in three replicates. Two P levels, i.e. 10 mg P $kg^{-1}$ soil (20 kg P $ha^{-1}$, low P) and 40 mg P per $kg^{-1}$ soil (80 kg P $ha^{-1}$, high P) were established with triple super phosphate (TSP). These levels of P were adopted from Wang et al. [16]. A basal dose of 40 mg N $kg^{-1}$ was adjusted with urea in all pots. An additional 40 mg N $kg^{-1}$ was supplied to each pot at tillering (after 30 days of sowing) and booting stage (after 60 days of sowing) of the crop to reach total amount of nitrogen 120 mg N $kg^{-1}$ soil (240 kg N $ha^{-1}$). All pots received 40 mg K $kg^{-1}$ soil (80 kg K $ha^{-1}$) in the form of sulfate of potash (SOP). Before seed sowing, a basal dose of urea and all of TSP and SOP were mixed well into the soil.

Ten seeds were sown in each pot, and at two-leaf stage the seedlings were thinned to maintain only five similar sized plants. Pots were irrigated with reverse osmosis (RO) water as and when required. At maturity, plants were harvested and separated into grain and straw by manual threshing. Grain and straw weight was measured using a weighing balance. The samples were then dried at 70 $^oC$ in a forced-air oven for three days. The oven-dried samples of grain and straw were finely ground and one gram of each sample was digested in a di-acid mixture of nitric acid ($HNO_3$) and perchloric acid ($HClO_4$) [17]. The digested material was analyzed spectrophotometrically for P contents following the method of Chapman and Pratt [18]. Phosphorus uptake (mg P $pot^{-1}$) in grain and straw was estimated by multiplying P concentration with their corresponding dry weights. Total P uptake (mg P $pot^{-1}$) was obtained by adding up grain and straw P uptake. The following parameters were calculated as described by Gill et al. [13]:

**Phosphorus stress factor (PSF %)** =

$$\frac{\text{Grain yield at high P (g pot}^{-1}) - \text{Grain yield at low P (g pot}^{-1})}{\text{Grain yield at high P (g pot}^{-1})} \times 100$$

**Phosphorus harvest index (PHI %)** =

$$\frac{\text{Grain P uptake (mg P pot}^{-1})}{\text{Total P uptake (grain+straw, mg P pot}^{-1})} \times 100$$

**Phosphorus physiological efficiency ratio (PPER) =**

$$\frac{\text{Grain yield (g pot}^{-1})}{\text{Total P uptake (grain+straw, g P pot}^{-1})}$$

**Phosphorus biological yield efficiency ratio (PBER) =**

$$\frac{\text{Total yield (grain+straw, g pot}^{-1})}{\text{Total P uptake (grain+straw, g P pot}^{-1})}$$

The collected data were analyzed by the analysis of variance (ANOVA) technique according to the two factorial-completely randomized factorial designs. The treatment means were differentiated by Tukey's Honestly Significant Difference (HSD) method [19] at 5% probability level. Moreover, each genotype was assigned an index score/value of 1, 2 or 3 for low (if mean is < μ - SD), medium (if mean is between μ - SD to μ + SD) and high (if mean > μ+SD) grade of performance of each character at each P level following the method of Gill et al. [13]. The μ and SD represent population mean and standard deviation, respectively. Total index score for each genotype at each P level was calculated by adding up the index values of all the characters for that particular genotype. In case of PSF, an opposite order of low and high index scores was applied because the genotypes with higher PSF values were more sensitive to low P stress.

## 3. RESULTS

### 3.1 Grain, Straw and Total Yield (g pot$^{-1}$)

The data analysis showed that the individual effects of genotypes and P levels on grain yield were significant ($P < 0.05$), but their interactive effects could not produce significant effect (Table 1). Averaging across the genotypes, grain yield increased from 6.79 to 7.82 g pot$^{-1}$ with increase in the P level from 10 to 40 mg P kg$^{-1}$ soil. The wheat genotypes demonstrated variable yield response at each P level. At low P level, the grain yield ranged between the highest (8.42 g pot$^{-1}$) for EST-28/11 and the lowest (5.46 g pot$^{-1}$) for MSH-5. At high P level, NIA-Sunder and MSH-5 produced the highest and the lowest grain yields, respectively. Increment in grain yield by increasing P level indicates the responsiveness of the genotypes, though the genotypes varied widely in their extent of responsiveness. Genotype AA-V2 exhibited the highest response to P as it registered 43% increase in grain yield when P rate was increased from 10 to 40 mg P kg$^{-1}$ soil. Genotypes AA-V1, MSH-3 and EST-28/11 exhibited no response to the higher level of P in soil.

Straw yield was significantly ($P < 0.05$) influenced by the individual as well as interactive effects of genotypes and P levels. Averaged across 10 genotypes, the straw yield increased from 14.2 to 16.3 g pot$^{-1}$ when P application rate increased from 10 to 40 mg P kg$^{-1}$ soil. The genotypes exhibited remarkable variations in straw yield at both P levels. The straw yield ranged from 9.66 to 17.5 g pot$^{-1}$ at low P level and from 13.3 to 18.7 g pot$^{-1}$ at high P level (Table 1).

Total yield (grain + straw) also increased with increase in the soil P level. About 12% increase in total yield was recorded by increasing P rate from 10 to 40 mg P kg$^{-1}$ soil. At low P level, the highest total yield (24.3 g pot$^{-1}$) was produced by ESW-9525 and it was statistically at par with that of EST-28/11 and NIA-Sunder, while AA-V2, AA-V1 and MSH-5 produced the lowest and statistically identical total yield. It ranged from 19.6 to 26.5 g pot$^{-1}$ at high P level. Like grain yield, no significant interactive effects of the genotypes and P levels could be observed on total yield (Table 1).

### 3.2 Phosphorus Stress Factor (PSF %)

Phosphorus stress factor (PSF) or relative tolerance to P deficiency varied significantly ($P < 0.05$) among wheat genotypes (Table 1). In this study, PSF ranged between 0.15 and 30.1 %, indicating an extensive genetic variability among the wheat genotypes for grain production in response to high P level. Genotypes viz., AA-V1, MSH-3 and EST-28/11 showed no response to high P level, as their PSF values were equal or lower than one. However, AA-V2, BWQ-4 and EST-29/9 with higher PSF values can be regarded as high P responsive.

### 3.3 Phosphorus Uptake (mg P pot$^{-1}$)

Phosphorus uptake in both grain and straw was significantly influenced by the main as well as interactive effects of the genotypes and P levels (Table 2). Phosphorus uptake in grain increased by 45% with the application of high P rate (40 mg P kg$^{-1}$ soil). At low P level, EST-28/11 with grain P

**Table 1:** Performance of wheat genotypes for grain yield, straw yield, total yield and phosphorus stress factor (PSF) at low and high P levels

| | Grain yield (g pot$^{-1}$) | | Straw yield (g pot$^{-1}$) | | Total yield (g pot$^{-1}$) | | PSF (%) |
|---|---|---|---|---|---|---|---|
| **Genotypes** | Low P | High P | Low P | High P | Low P | High P | |
| AA-V1 | 6.27 (2)† | 6.31 (1) | 12.5 (2) | 13.3 (1) | 18.8 (1) | 19.6 (1) | 0.67 (3) |
| AA-V2 | 6.17 (2) | 8.82 (2) | 9.66 (1) | 15.1 (2) | 18.5 (1) | 21.3 (1) | 30.1 (1) |
| AA-V3 | 6.99 (2) | 8.04 (2) | 13.0 (2) | 14.9 (2) | 20.0 (2) | 22.9 (2) | 12.8 (2) |
| MSH-3 | 7.95 (3) | 7.98 (2) | 14.6 (2) | 15.1 (2) | 22.6 (2) | 23.1 (2) | 0.38 (3) |
| MSH-5 | 5.46 (1) | 6.26 (1) | 13.7 (2) | 15.3 (2) | 19.1 (2) | 21.5 (2) | 12.7 (2) |
| BWQ-4 | 5.50 (1) | 7.54 (2) | 16.6 (3) | 18.3 (3) | 22.1 (2) | 25.8 (2) | 27.3 (1) |
| EST-28/11 | 8.42 (3) | 8.43 (2) | 15.5 (2) | 18.1 (2) | 24.0 (3) | 26.5 (3) | 0.15 (3) |
| EST-29/9 | 5.95 (2) | 7.51 (2) | 14.6 (2) | 17.8 (2) | 20.5 (2) | 25.3 (2) | 20.6 (2) |
| ESW-9525 | 6.87 (2) | 7.70 (2) | 17.5 (3) | 18.7 (3) | 24.3 (3) | 26.4 (3) | 10.9 (2) |
| NIA-Sunder | 8.33 (3) | 9.66 (3) | 14.6 (2) | 16.2 (2) | 23.0 (2) | 25.9 (2) | 13.5 (2) |
| $HSD_{0.05}$, P | 0.44 | | 0.58 | | 0.59 | | --- |
| $HSD_{0.05}$, G | 1.65 | | 2.16 | | 2.20 | | 16.05 |
| $HSD_{0.05}$, P×G | NS | | 3.45 | | NS | | --- |

† Values in parentheses represent index scores.
$HSD_{0.05}$ = Honestly Significant Difference at 5% probability level; P = phosphorus levels;
G = genotypes; P×G = interaction between phosphorus levels and genotypes; NS = non-significant

**Table 2:** Phosphorus uptake in grain and straw of wheat genotypes grown at low and high P levels

| | P uptake (mg P pot$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Grain | | Straw | | Total | |
| **Genotypes** | Low P | High P | Low P | High P | Low P | High P |
| AA-V1 | 22.0 (2)† | 30.6 (1) | 6.10 (2) | 7.40 (1) | 28.1 (2) | 38.0 (1) |
| AA-V2 | 19.9 (2) | 40.0 (3) | 3.70 (1) | 7.39 (1) | 23.6 (1) | 47.3 (2) |
| AA-V3 | 24.6 (2) | 35.8 (2) | 4.05 (1) | 10.8 (2) | 28.7 (2) | 46.6 (2) |
| MSH-3 | 30.9 (3) | 39.9 (3) | 8.64 (2) | 10.1 (2) | 39.5 (3) | 50.0 (2) |
| MSH-5 | 19.9 (2) | 31.3 (1) | 7.12 (2) | 10.6 (2) | 27.0 (2) | 41.9 (1) |
| BWQ-4 | 18.1 (1) | 36.0 (2) | 11.5 (3) | 13.3 (3) | 29.6 (2) | 49.3 (2) |
| EST-28/11 | 38.0 (3) | 41.7 (3) | 5.96 (2) | 10.1 (2) | 43.9 (3) | 51.7 (3) |
| EST-29/9 | 22.4 (2) | 33.7 (2) | 8.11 (2) | 12.7 (3) | 30.5 (2) | 46.4 (2) |
| ESW-9525 | 28.5 (2) | 34.3 (2) | 10.4 (3) | 12.0 (2) | 38.9 (3) | 46.3 (2) |
| NIA-Sunder | 23.9 (2) | 37.9 (2) | 4.87 (2) | 8.29 (2) | 28.8 (2) | 46.2 (2) |
| $HSD_{0.05}$, P | 1.92 | | 0.42 | | 1.81 | |
| $HSD_{0.05}$, G | 7.11 | | 1.57 | | 6.71 | |
| $HSD_{0.05}$, P×G | 11.38 | | 2.51 | | 10.74 | |

† Values in parentheses represent index scores.
$HSD_{0.05}$ = Honestly Significant Difference at 5% probability level; P = phosphorus levels;
G = genotypes; P×G = interaction between phosphorus levels and genotypes

uptake of 38.0 mg P pot$^{-1}$ was statistically superior to the rest of genotypes. Genotypes EST-28/11, MSH-3, AA-V2 and NIA-Sunder had the highest and statistically identical grain P uptake at high P level. Overall, P uptake in grain ranged between 18.1 and 38.0 mg P pot$^{-1}$ at low P level and 31.3 and 41.7 mg P pot$^{-1}$ at high P level.

Phosphorus uptake in straw ranged between 3.70 and 11.5 mg P pot$^{-1}$ at low P level and from 7.39 to 13.3 mg P pot$^{-1}$ at high P level depending on the genotypes. Increase in P application rate caused a 45% increase in P uptake in straw. Averaging across the genotypes, total P uptake (grain + straw) increased from 31.9 to 46.4 (45 % increase) by increasing P level in the soil. Total P uptake ranged between 23.6 to 43.9 mg P pot$^{-1}$at low P and 38.0 to 51.7 mg P pot$^{-1}$ at high P. Generally, EST-28/11 was the highest accumulator of P at both P levels (Table 2).

### 3.4 Phosphorus Efficiency Indices

Phosphorus harvest index (PHI) represents the portion of total plant P (grain + straw) present in the

**Table 3:** Phosphorus efficiency indices and total index scores of wheat genotypes at low and high P levels

| | PHI (%)[a] | | PPER[b] | | PBER[c] | | Total index score[d] | |
|---|---|---|---|---|---|---|---|---|
| **Genotypes** | Low P | High P | Low P | High P | Low P | High P | Low P | High P |
| AA-V1 | 78.2 (2)† | 80.5 (2) | 223 (2) | 167 (2) | 670 (2) | 517 (2) | 20 | 12 |
| AA-V2 | 84.1 (2) | 84.3 (3) | 261 (3) | 186 (2) | 789 (3) | 450 (1) | 17 | 17 |
| AA-V3 | 85.8 (3) | 76.8 (2) | 243 (2) | 172 (2) | 699 (2) | 496 (2) | 20 | 18 |
| MSH-3 | 78.2 (2) | 79.7 (2) | 201 (2) | 160 (2) | 571 (1) | 462 (1) | 23 | 18 |
| MSH-5 | 73.3 (2) | 74.3 (2) | 201 (2) | 149 (1) | 715 (2) | 528 (2) | 19 | 14 |
| BWQ-4 | 60.8 (1) | 72.8 (1) | 185 (2) | 152 (2) | 750 (2) | 525 (2) | 18 | 19 |
| EST-28/11 | 86.3 (3) | 80.5 (2) | 192 (2) | 163 (2) | 548 (1) | 513 (2) | 25 | 21 |
| EST-29/9 | 73.4 (2) | 72.5 (1) | 195 (2) | 162 (2) | 674 (2) | 548 (2) | 20 | 18 |
| ESW-9525 | 73.2 (2) | 74.0 (2) | 176 (1) | 166 (2) | 628 (2) | 573 (3) | 23 | 21 |
| NIA-Sunder | 82.9 (2) | 81.9 (2) | 289 (3) | 209 (3) | 801 (3) | 562 (3) | 23 | 21 |
| $HSD_{0.05}$, P | NS | | 5.15 | | 28.48 | | | |
| $HSD_{0.05}$, G | 6.27 | | 19.11 | | 105.71 | | | |
| $HSD_{0.05}$, P×G | 10.03 | | 30.58 | | 169.13 | | | |

[a]PHI, phosphorus harvest index; [b]PPER, phosphorus physiological efficiency ratio; [c]PBER, phosphorus biological yield efficiency ratio; [d]Total index score is the sum of individual index scores for each parameter for each genotype at each P level.
† Values in parentheses represent index scores.
$HSD_{0.05}$ = Honestly Significant Difference at 5% probability level; P = phosphorus levels;
G = genotypes; P×G = interaction between phosphorus levels and genotypes; NS = non-significant

grain at crop harvest. The P levels could not produce significant ($P < 0.05$) effects on PHI of genotypes; however, the genotypes exhibited considerable variability for PHI at each P level. Variations among wheat genotypes for P allocation to grains were also elucidated by significant genotype × P level interactions (Table 3). The PHI ranged from 60.8 to 86.3% at low P and 72.5 to 84.3% at high P. This means that wheat genotypes remobilized most of their accumulated P towards grain.

Phosphorus physiological efficiency ratio (PPER) indicated the gram of grain produced per gram of P accumulated in grain plus straw. It varied significantly ($P < 0.05$) among the wheat genotypes at both P levels (Table 3). Genotypic variability for PPER was also elaborated by the significant ($P < 0.05$) genotype × P level interaction. The PPER of wheat genotypes ranged from 176 to 289 and 149 to 209 at low and high P levels, respectively. It can be observed from the data that the genotypes demonstrated higher PPER at low P level, where P uptake was comparatively low, than at high P supply. This implies that increasing P application rate had negative effects on PPER. Of all genotypes, NIA-Sunder attained the highest PPER at both levels.

Phosphorus biological Efficiency ratio (PBER) measures the biological/total yield (grain + straw) produced in relation to total P uptake. The genotypes and P levels as well as their interactions significantly ($P < 0.05$) affected this index of P utilization efficiency (Table 3). Like PPER, the PBER also decreased with increasing P level. It varied from 548 to 801 and 450 to 573 in low and high P treatments, respectively.

### 3.5 Categorization of Genotypes on the Basis of Index Scores

The wheat genotypes were grouped into low, medium or high scoring based on the index scores of 10 characters at low P level (Table 4) and 9 characters at high P level (Table 5). The classification scheme was adopted from Gill et al. [13]. A perusal of data showed that most of the genotypes were generally in the medium category for various parameters at both P levels. For instance, on the basis of grain yield, two genotypes were classified into low (< 5.67 g $pot^{-1}$), 5 into medium (5.67-7.91 g $pot^{-1}$) and three into high (> 7.91 g $pot^{-1}$) scoring category at low P level (Table 4). At adequate P level, two genotypes were in low (6.78 g $pot^{-1}$), seven in medium (6.78-8.87 g $pot^{-1}$) and one in high (8.87 g $pot^{-1}$) grain yield group (Table 5). Based on total P uptake, 1, 6 and 3 genotypes were classified as low (< 25.3 mg P $pot^{-1}$), medium (25.3-38.4 mg P $pot^{-1}$) and high (> 38.4 mg P $pot^{-1}$) P uptake groups, respectively, at low P level. The corresponding values for total P uptake at adequate P level were 2, 7, and 1, respectively for low, medium, and high groups.

**Table 4:** Classification of wheat genotypes on the basis of index scores of various parameters at low P level. Each genotype was assigned an index score of 1, 2 or 3 for low (if mean is < μ - SD), medium (if mean is between μ - SD to μ + SD) and high (if mean > μ+SD) grade of performance of each character. The μ and SD represent population mean and standard deviation, respectively.

| | Low (score 1) | Medium (score 2) | High (score 3) |
|---|---|---|---|
| Grain yield ($g\ pot^{-1}$) | < 5.67<br>MSH-5 & BWQ-4 | 5.67-7.91<br>EST-29/9, AA-V2, AA-V1, ESW-9525 & AA-V3 | > 7.91<br>MSH-3, NIA-Sunder & EST-28/11 |
| Straw yield ($g\ pot^{-1}$) | < 12.0<br>AA-V2 | 12.0-16.4<br>AA-V1, AA-V3, MSH-5, EST-29/9, MSH-3, NIA-Sunder & EST-28/11 | > 16.4<br>BWQ-4 & ESW-9525 |
| Total yield ($g\ pot^{-1}$) | < 19.1<br>AA-V1 & AA-V2 | 19.1-23.5<br>MSH-5, AA-V3, EST-29/9, BWQ-4, MSH-3 & NIA-Sunder | > 23.5<br>EST-28/11 & ESW-9525 |
| Grain P uptake ($mg\ P\ pot^{-1}$) | < 18.7<br>BWQ-4 | 18.7-30.9<br>MSH-5, AA-V2, AA-V1, EST-29/9, AA-V3, NIA-Sunder & ESW-9525 | > 30.9<br>MSH-3 & EST-28/11 |
| Straw P uptake ($mg\ P\ pot^{-1}$) | < 4.43<br>AA-V2 & AA-V3 | 4.43-9.66<br>NIA-Sunder, EST-28/11, AA-V1, MSH-5, EST-29/9 & MSH-3 | > 9.66<br>ESW-9525 & BWQ-4 |
| Total P uptake ($mg\ P\ pot^{-1}$) | < 25.3<br>AA-V2 | 25.3-38.4<br>MSH-5, AA-V1, AA-V3, NIA-Sunder, BWQ-4 & EST-29/9 | > 38.4<br>ESW-9525, MSH-3 & EST-28/11 |
| PSF (%) | > 23.6<br>AA-V2 & BWQ-4 | 2.18-23.6<br>ESW-9525, MSH-5, AA-V3, NIA-Sunder & EST-29/9 | < 2.18<br>EST-28/11, MSH-3 & AA-V1 |
| PHI (%) | < 69.8<br>BWQ-4 | 69.8-85.5<br>EST-29/9, ESW-9525, MSH-5, MSH-3, AA-V1, NIA-Sunder & AA-V2 | > 85.5<br>AA-V3 & EST-28/11 |
| PPER | < 179<br>ESW-9525 | 179-254<br>BWQ-4, EST-28/11, EST-29/9, MSH-3, MSH-5, AA-V1 & AA-V3 | > 254<br>AA-V2 & NIA-Sunder |
| PBER | < 600<br>EST-28/11 & MSH-3 | 600-769<br>ESW-9525, AA-V1, EST-29/9, AA-V3, MSH-5 & BWQ-4 | > 769<br>AA-V2 & NIA-Sunder |

PSF, phosphorus stress factor; PHI, phosphorus harvest index; PPER, phosphorus physiological efficiency ratio; PBER, phosphorus biological yield efficiency ratio

Moreover, the wheat genotypes were grouped into six categories by regressing grain yield and total P uptake at low P level according to Gill et al. [13]. Two genotypes were placed in high grain yield-high P uptake (HGY-HP), three in medium grain yield-medium P uptake (MGY-MP), two in low grain yield-medium P uptake (LGY-MP) and one in each of high grain yield-medium P uptake (HGY-MP), medium grain yield-high P uptake (MGY-HP), medium grain yield-low P uptake (MGY-LP) group (Figure 1).

## 4. DISCUSSION

Productivity of low P input agricultural systems can be sustained by introducing crop cultivars efficient in P acquisition and/or utilization. Earlier research has shown that wheat genotypes differ in their response to P starvations [6, 13, 20, 21]. Genotypes selected on the basis of absolute grain yield in relation to P uptake, PHI, PPER, and PPBR will lead us to identify useful genetic characters which can be utilized in future breeding ventures aimed at evolving high-yielding P efficient cultivars. Such type of intervention would not only reduce the production costs, but also minimize P-associated environmental pollution hazards [22]. The present study was envisioned to identify ideal genotypes which have better adaptability to soil of varying P availability.

In this study, grain yield of AA-V1, MSH-3 and

**Table 5:** Classification of wheat genotypes on the basis of index scores of various parameters at high P level. Each genotype was assigned an index score of 1, 2 or 3 for low (if mean is < μ - SD), medium (if mean is between μ - SD to μ + SD) and high (if mean > μ+SD) grade of performance of each character. The μ and SD represent population mean and standard deviation, respectively.

| | Low (score 1) | Medium (score 2) | High (score 3) |
|---|---|---|---|
| Grain yield ($g\ pot^{-1}$) | < 6.78<br>MSH-5 & AA-V1 | 6.78-8.87<br>EST-29/9, BWQ-4, ESW-9525, MSH-3, AA-V3, EST-28/11 & AA-V2 | > 8.87<br>NIA-Sunder |
| Straw yield ($g\ pot^{-1}$) | < 14.4<br>AA-V1 | 14.4-18.1<br>AA-V3, AA-V2, MSH-3, MSH-5, NIA-Sunder, EST-29/9 & EST-28/11 | > 18.1<br>BWQ-4 & ESW-9525 |
| Total yield ($g\ pot^{-1}$) | < 21.3<br>AA-V1 & AA-V2 | 21.3-26.3<br>MSH-5, AA-V3, MSH-3, EST-29/9, BWQ-4 & NIA-Sunder | > 26.3<br>ESW-9525 & EST-28/11 |
| Grain P uptake ($mg\ P\ pot^{-1}$) | < 32.4<br>AA-V1 & MSH-5 | 32.4-39.9<br>ESW-9525, EST-29/9, AA-V3, BWQ-4 & NIA-Sunder | > 39.9<br>MSH-3, AA-V2 & EST-28/11 |
| Straw P uptake ($mg\ P\ pot^{-1}$) | < 8.18<br>AA-V2 & AA-V1 | 8.18-12.3<br>NIA-Sunder, EST-28/11, MSH-3, MSH-5, AA-V3 & ESW-9525 | > 12.3<br>EST-29/9 & BWQ-4 |
| Total P uptake ($mg\ P\ pot^{-1}$) | < 42.4<br>AA-V1 & MSH-5 | 42.4-50.3<br>NIA-Sunder, ESW-9525, EST-29/9, AA-V3, AA-V2, BWQ-4 & MSH-3 | > 50.3<br>EST-28/11 |
| PHI (%) | < 73.5<br>EST-29/9 & BWQ-4 | 73.5-81.9<br>MSH-5, ESW-9525, AA-V3, AA-V1, MSH-3, EST-28/11 & NIA-Sunder | > 81.9<br>AA-V2 |
| PPER | < 151<br>MSH-5 | 151-186<br>BWQ-4, EST-28/11, EST-29/9, MSH-3, AA-V1, AA-V3, ESW-9525 & AA-V2 | > 186<br>NIA-Sunder |
| PBER | < 478<br>AA-V2 & MSH-3 | 478-557<br>AA-V3, EST-28/11, AA-V1, BWQ-4, MSH-5 & EST-29/9 | > 557<br>NIA-Sunder & ESW-9525 |

PHI, phosphorus harvest index; PPER, phosphorus physiological efficiency ratio; PBER, phosphorus biological yield efficiency ratio

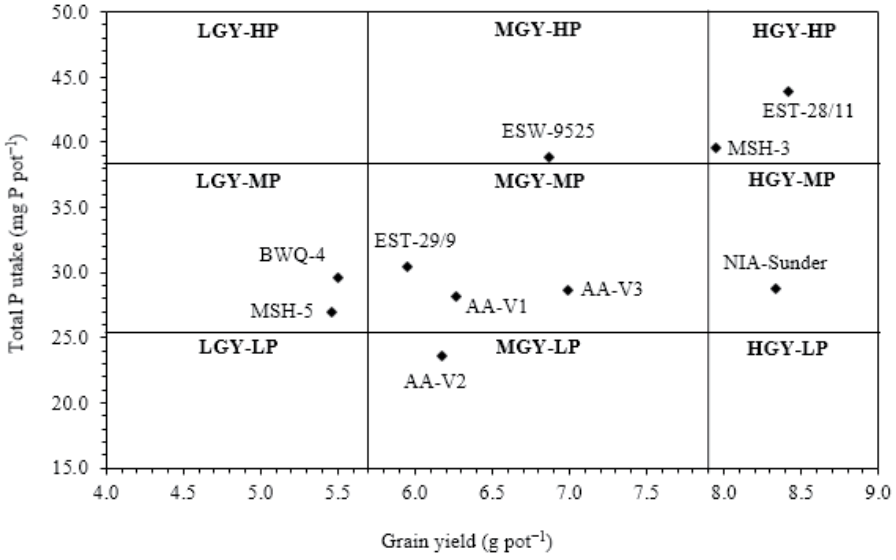


**Fig. 1.** Categorization of 10 wheat genotypes into different groups on the basis of grain yield and total P uptake at low P level according to Gill et al. [13]. LGY, MGY and HGY represent low, medium and high grain yield, respectively, while, LP, MP and HP represent low, medium and high P uptake, respectively.

EST-28/11 was not inhibited by P deficiency which indicated that these genotypes had a potential to sustain growth and development in P-starved environments. Yaseen and Malhi [23] have also reported that the present day wheat cultivars have ability to produce more than their older counterparts even at lower rates of P application. Genotypes viz., MSH-3 and EST-28/11, having least sensitivity to P deficiency, overcame the low P stress by allocating a major chunk of plant P in grain which is evident from their high grain as well total P uptake in low P treatment (Table 2). This information pointed out an increased translocation of absorbed P towards grain.

Grain yield of genotypes coincided much with total P uptake, though with few exceptions. For example, at deficient P supply, ESW-9525 with high P uptake characteristics was in the medium-grain-yield category and NIA-Sunder having a medium P uptake was categorized as high-grain-yielder (Table 4). Grain yield also had a strong positive relationship ($r > 0.65$, $P < 0.001$) with grain P uptake at both P levels, which indicates that genotypes efficient in accumulating more P in their grain also produced higher grain yield.

The PHI refers to the translocation of absorbed P towards grains. In the present study, the higher P level could not increase the pace of P translocation from straw to grain. Moreover, a strong relationship between grain yield and PHI ($r > 0.61$, $P < 0.001$) revealed that the genotypes having ability to translocate more of the accumulated P towards grains had higher grain yields at both P levels. The strong positive relationship of grain yield with grain P uptake ($r > 0.82$, $P < 0.001$) at both P levels also supported this argument. However, the higher PHI may result in accelerated P mining from the farmlands [24, 25] which can negatively affect the sustainability of farming systems. Breeding for low grain P concentration can be a viable option to minimize P loss; however, such intervention may have a negative effect on seed viability as well as its nutritional value. Therefore, a minimum P concentration in grain needs to be maintained for sustaining seed viability and crop establishment. High yields without reducing grain P concentration can be achieved by improved translocation of P into grains in addition to increased P uptake. In this study, 78% of total accumulated P was found in wheat grain at harvest (Table 3), showing a limited scope for increased translocation of P from straw into grain. Considering 70 to 80% of total P is present in grain, selecting for higher grain yield will further dilute grain P concentration as long as P uptake efficiency is not improved [26].

The PPER and PBER depict the efficiency by which plants utilize the accumulated P to produce grain and aboveground biomass, respectively, and they have been successfully used by Korkmaz et al. [27] and Yaseen and Malhi [23, 26] for evaluating PUE of wheat genotypes. An ideal genotype tends to absorb more P, apportions more P into grain and produces more grain per unit of absorbed P. In this study, genotypic variability for PPER at each P level indicates differential P utilization efficiency of these genotypes. It was evident from PPER results that the high-yielding genotype NIA-Sunder with high value for PPER at both P levels was efficient in P utilization and can yield well in low as well as high P environment. A positive relationship between grain yield and PPER at low ($r = 0.35$, $P > 0.05$) and high P level ($r = 0.72$, $P < 0.001$) indicated that some genotypes adopted similar physiological mechanisms to produce grain. At low P level, genotypes with high values of PBER were generally characterized with low grain yield as well as low grain P accumulation. Hence, these genotypes were inefficient in P use. This was also reflected by their relatively high values of PSF.

The results of our study highlighted that the genotypes formed six groups on the basis of grain yield and total P uptake at low P level. Such type of classification would help in identification of wheat genotypes for growing on P deficient soils and selection of parents for recombination breeding to develop P efficient cultivars [22]. Three genotypes viz., EST-29/9, AA-V3 and AA-V1 were placed in MGY-MP (medium grain yield-medium P uptake) group with a total index score of 20 for each genotype. Genotypes EST-28/11 and MSH-3, with respective index scores of 25 and 23, were placed in HGY-HP category. These genotypes were efficient in P acquisition as well as utilization for grain production under low P availability and can be selected for soils with a wide range of P contents [22, 23, 26]. Genotype NIA-Sunder was high grain yielder with a medium P accumulation (HGY-MP). The LGY-MP group comprised BWQ-4 and MSH-

5. Genotypes ESW-9525 and AA-V2 were members of MGY-HP and MGY-LP groups, respectively. Moreover, these findings suggest that some of the P-efficient genotypes viz., EST-28/11 and MSH-3 were more effective in P uptake from low P medium, and that P uptake was the primary process contributing to P efficiency for these genotypes. On the other hand, NIA-Sunder efficiently utilized the absorbed P, although the amount of absorbed P was not high. Efficient P utilization seemed to be the dominant phenomenon underlying P efficiency in this context. Wheat genotypes from various groups in this study should be used for examining shoot and root traits responsible for high P uptake, grain yield and translocation of P from different plant parts to grain sink. Earlier studies have revealed that P efficiency traits in wheat are inheritable and can be exploited in breeding programs destined for improving P efficiency [16, 28].

## 5. CONCLUSION

The wheat genotypes examined in this study demonstrated significant variations for grain yield and P uptake and utilization. Such genetic diversity can be useful to develop genotypes with improved genetics for P efficiency. The genotypes EST-28/11, MSH-3 and NIA-Sunder were efficient in grain production and/or P uptake; hence, they can be grown successfully on P deficient soils. Moreover, inter-mating between the wheat genotypes belonging to different groups (e.g. HGY-HP and HGY-MP) will further expand genetic variation for grain yield and P uptake.

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Research Article

# Exploring the Rhizospheric Bacterial Communities of *Mangifera indica*

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**Abstract:** Rhizosphere soil plays an important role in providing environment conducive for growth of plants, therefore the knowledge about its living constituents is of paramount importance. The present study has partially determined the dynamics of bacterial communities in the rhizosphere soil of *Mangifera indica*, an indigenous fruit tree. Soil samples from the rhizosphere of *Mangifera indica* were collected from various locations and analysed for bacterial load and communities. The experiments were performed in two distinct phases under two different growth conditions such are aerobic and anaerobic. The data of aerobic phase of this study revealed presence of spore forming and non-spore forming aerobic bacterial species belonging to *Bacillus, Enterobacter, Pseudomonas, Proteus,* and *Serratia genera*, while the findings of anaerobic phase yielded members of genus *Lactobacilli* only. *Lactobacilli* occupied an average 17% of total anaerobic communities of bacteria in the rhizosphere of *Mangifera indica*, while aerobic bacilli occupied only 8.33% of total aerobic bacterial communities. Data indicated that the rhizosphere soil of *Mangifera indica* is rich in bacterial communities belonging to both aerobic and anaerobic groups. However, the load of bacterial isolates varied dramatically from sample to sample suggesting that in addition to type of plant other factors such as soil environment and nutrients may influence the bacterial communities in the rhizosphere soil of a plant.

**Keywords:** Rhizosphere, soil, Bacterial communities, *Mangifera indica.*

## 1. INTRODUCTION

Rhizosphere is a compartment where soil is under the direct influence of plants via their roots. The organic compounds such as amino acids, proteins, fatty acids and flavonoids are usually secreted by plant roots in its rhizosphere soil which in turn shape the colonization of microbial communities (fungal, archeal and bacterial) in this compartment. These microbial communities of rhizosphere are collectively called as rhizobiome [1]. The rhizobiome has critical role in the nutrient cycling by assisting the plant in the uptake of several vital nutrients, such as phosphorous, potassium and nitrogen from the soil. The type of plant and soil affects the community structure of the rhizobiome. In addition, various environmental factors including regional climate and pollutants have been reported to influence the structure and diversity of bacterial communities of the rhizobiome [2]. *Mangifera indica*, commonly called as mango belongs to genus *Mangifera,* of the flowering plant family Anacardiaceae. It has been produced in tropical Asia and Pakistan is its fifth largest producer in the world. It has also been exported from Pakistan to different regions globally.

The bacterial communities of the rhizobiome have been shown to play a vital role in development of a plant by production of molecular signals. These signals act as communication tools for interaction between plant roots and root bacteria. This interaction exerts considerable beneficial influence upon fitness and development of the plant [3]. One of the best examples of plant fitness by bacterial signals, is the production of iron-binding compounds (siderophores) by genus *Pseudomonas* which determines quality of the growth of plant and responsible for more yield of plant [4]. Moreover, Pseudomonas spp. may cause suppression of some plant diseases [5]. It is also well known that plants have significant influence on the diversity,

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spatial distribution and abundance of soil microbes through the rhizospheres. It has been suggested that measurement of the microbial community structure of soil indicates the status of a system and help in understanding the ecological process [6].

A previous study has shown that plant species and soil type significantly affect the structure of Pseudomonas and Bacillus communities of rhizobiome [7]. In this context, the root microbiome of *Arabidopsis thaliana* plant was found more diverse than the bulk soil indicating that a plant genotype determines the pattern of colonization of specific microbial community inhabiting in roots [8]. Therefore, a better understanding of the microbial ecology of the rhizosphere of a plant may allow discovering potentially useful secondary metabolites for exploiting them as antagonists of pathogens. The present study was devised to assess the dynamics of bacterial communities in the rhizobiome of indigenous *Mangifera indica* (mango tree) growing under the common conditions of agriculture practices.

## 2. MATERIAL AND METHODS

### 2.1 Sources of Samples and their Collection

Soil samples were collected from the rhizosphere of indigenous mango tree cultivated at seven distinct sites of Jamshoro, Sindh. Samples were collected in a sterile beaker or flask with sterile spoon and immediately covered with cotton plugs or aluminum foil to avoid environmental contamination. Rhizosphere soil adhered to the roots of plant was collected by hand shaking off the soil against the sides of beaker as described previously [9]. The samples were transferred immediately to the laboratory for microbiological analysis.

### 2.2 Isolation of Aerobic Bacteria from Rhizosphere Soil Samples

One gram of soil sample was mixed into 5ml of sterile nutrient broth (OXOID, England). The tubes were incubated overnight aerobically. Following the incubation (enrichment stage), ten folds serial dilutions in sterile nutrient broth were made [10]. The last 3 dilutions ($10^{-2}$, $10^{-3}$, and $10^{-4}$) were pipette out and poured onto nutrient agar (OXOID, England) and then incubated overnight at 37°C aerobically. After incubation, discrete, and morphologically distinct colonies were streaked onto fresh nutrient agar to obtain pure culture colonies.

### 2.3 Isolation of Anaerobic Bacteria from Rhizosphere Soil Samples

For isolation of anaerobic bacteria from rhizosphere soil samples de Man Rogosa and Sharpe (MRS) medium was used [11]. One gram of soil sample was mixed into 5ml of sterile MRS broth (OXOID, England) and incubated for 3 days anaerobically. Following the incubation (enrichment stage), ten folds serial dilutions was made using 0.85% NaCl solution. The last 3 dilutions ($10^{-7}$, $10^{-8}$, and $10^{-9}$) were pipette out and poured onto MRS agar (OXOID, England) plates supplemented with 1% $CaCo_3$, and then incubated for 3-5 days at 37°C anaerobically. After incubation, discrete colonies with a halo zone around them were streaked onto fresh MRS agar plates to obtain pure culture colonies.

### 2.4 Identification of Bacterial Isolates

The isolated bacteria were identified by conventional methods including Gram staining, colonial/cultural, microscopic and biochemical characteristics i.e. fermentation of lactose, $H_2S$ production, ability to produce indole, citrate utilization, urease production, motility of organism and ability to produce cytochrome oxidase enzyme. Gram's staining was performed according to standardized method [12]. Spore staining was performed according to Schaeffer-Fulton method [13].

## 3. RESULTS

### 3.1 Bacterial Load in the Rhizosphere Soil of *Mangifera indica*

The bacterial Load analysis of culture-able aerobic and anaerobic bacteria of the rhizosphere soil samples was determined by counting colony forming units (CFU) (Table 01). Varied CFU count was observed from the samples, suggesting that the type of plant is not only factor influencing microbiome of plants; however other factors of soil also have influential role.

### 3.2 Bacterial Community Dynamics of the Rhizosphere of *Mangifera indica*

The data of Gram staining reaction performed on all bacterial isolates (n=60) of aerobically processed samples showed that proteobacteria were numerically dominant with average 90% of total bacteria. Some samples also yielded the growth of Firmicutes. In order to understand the diversity and community dynamics of bacteria, large creamy colonies grown anaerobically on MRS agar plates (n=100) from each of the samples were selected and subjected to microscopic observation using Gram's staining. It was found that 17% isolates in average were non spore forming Gram-positive bacilli. Whereas, colonies of aerobic bacteria showing distinct morphology on nutrient agar (n=60) were randomly selected for colonial characteristics (Table 02). The data demonstrated that they include Gram-positive bacilli, Gram-positive cocci, and Gram-negative bacilli (Fig 01). All aerobic and anaerobic isolates of this study were further identified on the basis of biochemical tests (Table 03). The results of biochemical characteristics of Gram-negative isolates (Table 04) showed that the bacterial communities of rhizosphere soil comprised mainly *Serratia* spp, *Enterobacter* spp, and *Pseudomonas* spp, *Acinetobacter* spp, and *Proteus* spp while Gram positive isolates included mainly *Bacillus* species (Table 05). The *Enterobacter* species were found dominant among the bacterial communities of rhizosphere soil of *Mangifera indica* plants. Furthermore, *Bacillus* isolates were capable of spore formation (Fig 02).

**Table 1** Colony forming units (CFU) of bacteria grown on MRS agar and Nutrient agar from the rhizosphere soil samples **.**

| Soil samples | CFU/g of soil on MRS agar (Anaerobic)[a] | Log10 of CFU/g of soil on MRS agar | CFU/g of soil on NA (Aerobic)[b] | Log10 of CFU/g of soil on NA |
|---|---|---|---|---|
| S1 | $9.5\times10^7$/ml | 7.977 | $2.0\times10^6$/ml | 6.301 |
| S2 | $7.9\times10^7$/ml | 7.897 | $6.8\times10^6$/ml | 6.832 |
| S3 | $2.9\times10^6$/ml | 6.462 | $6.3\times10^6$/ml | 6.799 |
| S4 | $1.32\times10^6$/ml | 6.120 | $3.4\times10^6$/ml | 6.531 |
| S5 | $1.24\times10^6$/ml | 6.093 | $2.4\times10^6$/ml | 6.380 |
| S6 | $1.6\times10^7$/ml | 7.204 | $9.0\times10^6$/ml | 6.954 |
| S7 | $2.0\times10^7$/ml | 7.301 | $6.4\times10^7$/ml | 7.806 |

[a] Plates incubated in anaerobic conditions
[b] Plates were incubated in aerobic conditions

**Table 2** Colonial characteristics of aerobic bacterial isolates grown on Nutrient agar

| Isolates Label | Colony morphology | Color | Elevation | Surface | Size |
|---|---|---|---|---|---|
| NA01- 06 | Irregular | Yellowish | Flat | Rough | Large |
| NA07-19 | Circular | Grayish white | Raised | Mucoid | Moderate |
| NA20- 25 | Irregular | Yellow green translucent | Flat | Smooth | Large |
| NA26- 46 | Circular | White creamy | Convex | Mucoid | Small-moderate |
| NA47- 51 | Circular | White | Umbonate | Mucoid | Small |
| NA52- 60 | Irregular | Opaque | Flat | Smooth | Large |

**Table 3** Biochemical characteristics of aerobic and anaerobic isolates of this study

| Isolates | Gram's staining reaction | Catalase test | Oxidase test | Spore staining | Vancomycin sensitivity |
|---|---|---|---|---|---|
| MRS1-16 | + | - | - | - | + |
| MRS17 | + | + | - | - | + |
| NA1-5 | + | + | - | + | - |
| NA06 | + | + | + | - | - |
| NA07-19 | - | + | - | - | - |
| NA20-25 | - | + | + | - | - |
| NA26-51 | - | + | - | - | - |
| NA52-60 | - | + | + | - | - |

**Table 4** Biochemical characteristics of aerobic Gram-negative isolates

| Isolates | TSI | | | Simmon's citrate | Urease | $H_2S$ | Indole | Motility |
|---|---|---|---|---|---|---|---|---|
| | Butt | Slants | Gas | | | | | |
| Type 1 isolates | Acidic | Acidic | - | + | - | + | + | + |
| Type 2 isolates | Alkaline | Alkaline | + | + | - | - | - | + |
| Type 3 isolates | Acidic | Acidic | - | + | - | - | - | + |
| Type 4 isolates | Acidic | Alkaline | - | + | + | - | | + |
| Type 5 isolates | Alkaline | Alkaline | - | + | - | - | - | + |

**Table 5** Types of aerobic bacteria isolated from the rhizosphere soil of *Mangifera indica*

| Type of Bacteria | Occurrence (% of aerobic bacteria) |
|---|---|
| *Bacillus* species | 8.33 |
| *Micrococcus* species | 1.66 |
| *Citrobacter* species | 21.66 |
| *Pseudomonas* species | 10 |
| *Enterobacter* species | 35 |
| *Serratia* species | 8.33 |
| *Proteus* species | 15 |

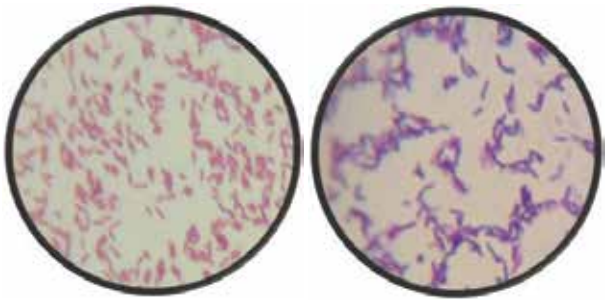

**Fig. 1.** Representative result of microscopic characteristics of aerobic Gram-negative (left) and Gram-positive (right) bacteria isolated from rhizosphere soil of *Mangifera indica* plant.

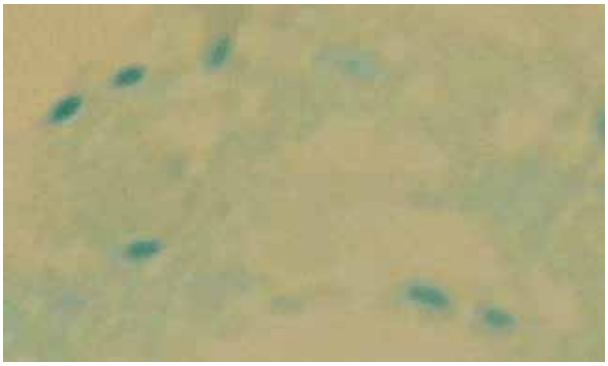

**Fig. 2.** Spore staining test result of aerobic *Bacillus* spp. isolated in this study.

*Lactobacilli* are LAB, because they produce lactic acid as an end product of fermentation of sugar on MRSA medium. Upon addition of $CaCO_3$ in media, lactate react with it and forms Ca-lactate as aresult a clear area/zone appears around the colonies of LAB. The appearance of a clear zone around the bacterial colony was considered as indicative of lactic acid production by the LAB isolate (Fig 03). Vancomycin sensitivity test has been suggested to differentiate between *Lactobacilli* species [14] since some *Lactobacilli* species (*L. rhamnosus*) are naturally resistant to vancomycin due to presence of D-Lac amino acid in place of D-ala in their cell wall structures. The present study has shown that majority of Lactobacilli isolates were sensitive to vancomycin (Fig 04) suggesting that the isolated strains were presumably *L. acidophilus* [14], and not *L. rhamnosus* which are naturally resistant to vancomycin [15].

## 4. DISCUSSION

Rhizosphere soil accommodates numerous bacterial communities due to its direct interaction with plant roots. Bacterial community structure of the rhizosphere soil of plants plays vital role in growth of the plant. However, the composition and quality of soil is debated. Since plant roots secrete many organic nutrients required for processing and functioning of these bacterial communities, they proliferate and propagate at their extreme. Alternatively, bulk soil lacks sufficient nutrients for

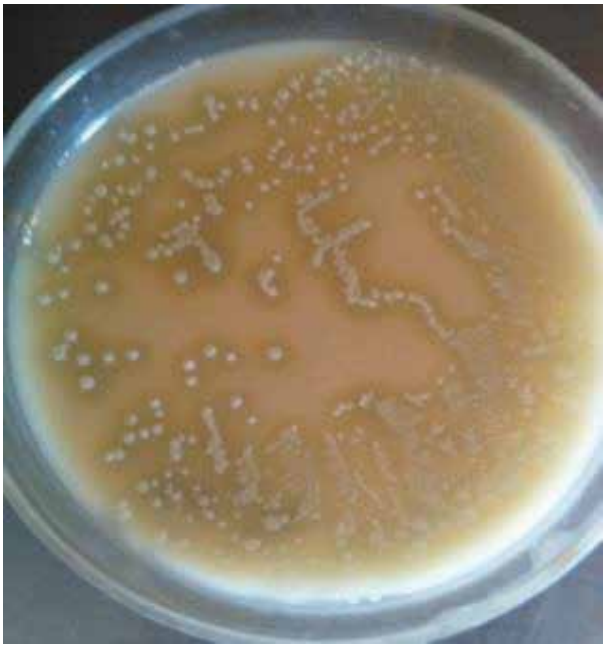

**Fig. 3.** MRS agar supplemented with $CaCO_3$ showing the lactic acid production by anaerobic *Bacilli* isolates. A clear zone around the bacterial colonies was considered as indicative of lactic acid production.

**Fig. 4.** Vancomycin sensitivity test to differentiate between *Lactobacilli* species. Some *Lactobacilli* species are naturally resistant to Vancomycin.

the growth of bacteria. A number of studies have investigated bacterial community dynamics of rhizosphere soil including in-depth study [16-18], analysis of such communities in rhizosphere soil of indigenous plant *Mangifera indica* is of paramount importance for recovering bacterial strains capable of performing unique functions including production of novel antibacterial compounds.

In a recent study, it has been shown that plant species and pH of soil may affect the microbial community structure in the rhizosphere soil [19]. Therefore, the present study was conducted to determine the influence of local weather and environment of soil on the rhizopheric microbiome of *Mangifera indica,* an important and second largest produced fruit in Pakistan. Knowing the bacterial population of the rhizosphere of *Mangifera indica* plant would be of greatest interest for understanding microbial ecology of rhizosphere of indigenous plants as well as it may help in planning future strategies to increase yield of this plant. Findings of this study can be exploited in discovery of biocontrol agents to cope with pathogens of this plant. In this study we observed that 17% of anaerobic population comprised Lactobacilli while others were not identified in this study. However, the communities of aerobic bacteria were of different types of Gram-positive and Gram-negative bacteria. It was found that the microbial load varied from sample to sample suggested that other factors of soil environment affect the microbial community dynamics of rhizosphere soil apart from plant type since all the samples in this study were collected from same type of plant.

## 5. CONCLUSION

In conclusion, knowing about bacterial communities of rhizosphere soil of particular locality is significant for ecosystem and the data obtained in this study provided insights into active bacterial population of *Mangifera indica* plant which may lead discovery of novel bacterial strains capable of providing an opportunity to treat challenging plant pathogens.

## 6. ACKNOWLEDGEMENTS

Authors are thankful to all gardeners of University of Sindh, Jamshoro for their help and cooperation during sampling process.

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Pakistan Academy of Sciences

## *Obituary*

# Mr. Tajammul H. Hashmi (1924–2018)

Mr. Tajammul H. Hashmi was born on Ist January 1924, in Gujrat, British India. Mr. Hashmi earned his MS degree from Michigan State University, USA in 1948. His main research areas were bridge design, Road Transport, Utilization of Sub-surface space, Control of water logging Salinity and Soil Mechanics.

Mr. Hashmi was Inter-Regional Advisor, United Nations Department of Technical Cooperation for Development, 1981-1991. Federal Secretary, Government of Pakistan, 1971-1973; Assistant Chief Engineer, Ministry of Railways and Communication, Government of Pakistan, 1949-1970.

Mr. Hashmi was honored with two Civil Awards in recognition to his services by Govt. of Pakistan. He was Past President and Fellow of Institute of Engineers Pakistan; Fellow, American Society of Civil Engineers, Executive Member of International Road Federation. Mr. Hashmi was elected as Fellow Pakistan Academy of Sciences in 1977.

On 24th May 2018, Mr. Hashmi left us all to meet his creator. We will miss him; so will his family. May Allah (SWT) bless him with highest ranks in Jannah. Ameen

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3. Kay, R.R. & C.R.L. Thompson. Forming patterns in development without morphogen gradients: differentiation and sorting. *Cold Spring Harbor Perspectives in Biology* 1: doi: 10.1101/cshperspect.a001503 (2009).

b. **Books**

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5. Alon, U. & D.N. Wegner (Ed.). *An Introduction to Systems Biology: Design Principles of Biological Circuits*. Chapman & Hall/CRC, Boca Raton, FL, USA (2006).

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6. Sarnthein, M.S. & J.D. Stanford. Basal sauropodomorpha: historical and recent phylogenetic developments. In: *The Northern North Atlantic: A Changing Environment.* Schafer, P.R. & W. Schluter (Ed.), Springer, Berlin, Germany, p. 365–410 (2000).
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