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Trichoderma, photo by Scot Nelson

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Book:

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Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Impact of bacterial consortium on plant growth development, fruit yield and disease resistance in tomato (*Solanum lycopersicum*)

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Abstract. *Mir RA, Ahmad MK, Bhat KR. 2023. Impact of bacterial consortium on plant growth development, fruit yield and disease resistance in tomato (*Solanum lycopersicum*). Asian J Trop Biotechnol 20: 1-9.* Agriculture plays an important role in the economic development of a country. However, using traditional fertilizers, disease resistance, and scarcity of nutrients have led to huge losses in plant productivity worldwide. *Bacillus* (*Bacillus megaterium*, *Bacillus siamensis*) are cosmopolitan species widely used as Plant Growth-Promoting Rhizobacteria (PGPR). This genus may be used along with other biocompatible microbes, including *Azotobacter* and *Trichoderma*, which can be used as consortia microbes as biofertilizers. However, the doubt remains in farmers' minds whether these biofertilizers completely replace chemical fertilizers. To gain insight into this doubt and clarify it, our study in this article is based on using Bio-NPK as an alternative to chemical fertilizers and *Trichoderma viride* as a bio-control agent. The bacterial strains and *T. viride* used in this experiment were isolated from GloBils organic farm. The plant growth parameters were measured after every 15 days. Fruit yield, weight, size, root length, root biomass, and shoot biomass were measured and compared with the control. Data analysis revealed a considerable difference between Bio-NPK-treated plants and chemical NPK-treated plants; further, a considerable difference is found in pots treated with Bio-NPK and *Trichoderma*. The Bio-NPK and *Trichoderma* showed higher disease resistance, stress tolerance, root development, biomass, and crop yield than chemical NPK. This study shows that Bio-NPK immunizes plants by re-organizing root development. At the same time, rhizobacteria stimulate defense response and simultaneously protect themselves from diseases.

Keywords: Bio-control, Bio-NPK, PGPR, soil application, *Trichoderma viride*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most popular and widely consumed vegetable crops worldwide, and high-quality yield is an essential requirement for its economic success. It is one of the most consumable vegetables in the Asian Sub-continent, constituting about 8% of the total land under vegetable crops and 9% of the total vegetable production. In India, Tomato occupies the third position after Potatoes and Onion but ranks second after potatoes worldwide. India is the second largest tomato-producing country and ranks second in the area and the production of tomatoes. There is an increase from 596.0 thousand ha in 2006-07 to 865.0 thousand ha in 2010-11. While in terms of production, it has increased from 10,055.0 to 16,826.0 thousand tons. The current production of tomatoes does not meet the demand for its consumption due to soils in the Asian subcontinent losing their nutrient and mineral contents, adversely affecting plant growth and quality. In addition, chemical fertilizers directly affect the production yield and imbalance the soil microbiome.

The incorrect application of chemical fertilizers affects soil health and declines the availability of essential nutrients. Furthermore, inappropriate use of inorganic fertilizers leaves soil phosphorus deficient. Therefore, the Phosphate Solubilizing Bacteria (PSB) from the rice rhizosphere was isolated and characterized (Gupta and Sharma 2018).

Among different types of Plant Growth-Promoting Rhizobacteria (PGPR), *Azotabacter*, and *Bacillus* species, *Bacillus megaterium* and *Bacillus siamensis* constitute an important class. These bacteria act as growth promoters by enhancing the uptake of Phosphorus (P) and Nitrogen (N). Phosphate Solubilizing Rhizobacteria (PSRB) plays an important role in maintaining soil health. Interaction of microbes with plants and roots occurs through root colonization. Plants get multifold benefits from this interaction in terms of soil fertility and increased growth leading to a fully developed plant body (Widawati and Suliasih 2006; Parray et al. 2016). Plant microbe interactions regulate different geochemical and biophysical processes within the soil (Dutta and Podile. 2010).

The PGPR present around the plant rhizosphere release growth promoting substances that help in the overall development of a plant. (Qiao et al. 2017). Root exudates containing sugars and amino acids attract rhizospheric microbes to synthesize different growth-promoting phytohormones (Bais et al. 2004; Ortíz-Castro et al. 2009). The root exudates play an important role in the survival of rhizosphere microorganisms in nutrient-deficient conditions (Barnawal et al. 2019; Khan et al. 2020). By dissolving phosphorous and potassium, PGPR produces phytohormones, such as auxins (IAA) and cytokinins. This phytohormone will enhance the plants' developmental processes and impart disease resistance. (Saleem et al. 2007; Pérez-Montaño et al. 2014; Khanna et al. 2019a,b,c; Bhat et al. 2020; Yaseen et al. 2020). PGPR around the

cereal rhizosphere helps plant species' overall growth and development (Mehnaz et al. 2010; Zhang et al. 2012; Teng et al. 2018; Chawngthu et al. 2020). In recent years, bio-fertilizer products have emerged as an essential component in integrated nutrient management and hold great promise to improve the yield and quality of crops (Wani et al. 1995). *Trichoderma* acts as biocontrol on plant foliage (Harman et al. 2004). In many situations, combining organic and inorganic fertilizers has produced higher yields than alone (Blackshaw 2005).

Plant growth-promoting microbes play a very significant role in regulating the dynamics of various processes, such as the decomposition of organic matter, the accessibility of various nutrients of plants such as iron, magnesium, nitrogen, potassium, and phosphorus, and promote the growth of the plants (Lalitha 2017). Moreover, bio-fertilizers (part of sustainable agriculture) are the best solution for the above problems (conventional agriculture). That is because bio-fertilizers are promising, eco-friendly, cost-effective, and more economical. Moreover, bio-fertilizers fix the substantial amount of atmospheric nitrogen in the soil, improve plant growth and productivity, and help in phosphorus availability and absorption, which plays a pivotal role in growth and productivity.

Bio-fertilizers combine the advantages of recycling organic waste, introducing beneficial microbes, and providing organic material that will create additional niches for beneficial indigenous microbes (Qiu et al. 2012; Fu et al. 2017).

MATERIALS AND METHODS

Study area

The study area was selected in districts Pulwama, Jammu and Kashmir, India, with locations and coordinates as shown in Figure 1.

Plant material and bacterial culture

Tomato seeds were obtained from Agricultural office Litter, Pulwama, Jammu, and Kashmir, India. All seeds were good quality hybrid seeds. All bacterial cultures (B10AR5, B54BM3, B50BS4) and fungal culture (BF6V30) were isolated from Apple Rhizospheric soil (Chandian Pajan, Kulgam, Jammu and Kashmir, India) and characterized for its nitrogen fixing and Plant growth promoting ability by following tests using standard protocols: Growth in Jensen's media, Nitrogen fixation efficiency by Kjeldahl method auxin production, biofilm formation, phosphate solubilization and siderophore production (Meyer and Abdallah 1978; Bric et al 1991; Nautiyal 1999; Morikawa et al. 2006). The isolated cultures were maintained in King's B Agar (KBA) medium, and the glycerol stock of the culture was stored at -80°C.

Seeds grown in invitro culture for pot trial

Tomato seeds were grown in plant tissue culture in invitro conditions using MS media to generate disease-free saplings and prevent any association with microbes before transferring them into pots for trial (Figure 2). Pots were filled with soil, sand, and compost in a ratio of 1:1:1 and dried at 100°C for 3 hrs in a Hot Air oven to avoid contamination in the soil. Pots filled with soil were used for the transplantation of saplings.

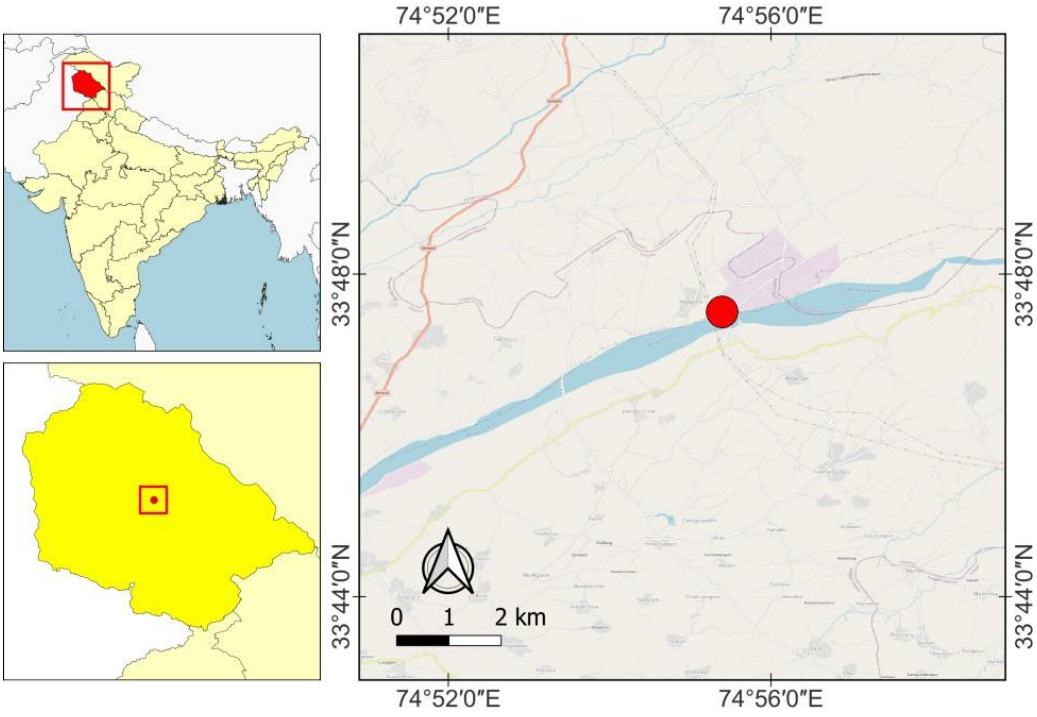


Figure 1. The location of GloBiL's Agri and Food Enterprises IGC Lassipoora Pulwama, India, indicates the field study. Field spot 1. (33°47'32.4"N 74°55'23.1"E)

Figure 2. Invitro growth of tomato seeds using MS media

Bacterial cultures with plant growth-promoting ability

The plant growth-promoting ability of bacterial cultures was tested using tomato plants grown in Plant tissue culture under controlled conditions and acclimatized in sterile soil under a greenhouse. Tomato seeds were sterilized in (70% ethanol for 5 mins and 0.1% mercuric chloride for 2-3 mins) before inoculation in plant tissue culture media (MS media). The growth-promoting ability of bacterial cultures was also checked under normal field conditions in a polyhouse. Tomato saplings grown in plant tissue culture media in test tubes were transferred into pots with two saplings in each pot in quadruplets containing sterile soil rite with soil: compost: sand in a 1:1:1 ratio and were kept in the greenhouse. The pot's moisture content was maintained by routine irrigation with the same amount of sterile water.

Tomato pot trial experiment

The tomato pot trial experiment was carried out inside a greenhouse under controlled conditions wherein saplings grown in plant tissue culture were transferred into pots with sterile soil rite. Eight different combinations were prepared with quadruplets of each combination and labeled accordingly. The pots were labeled as TT1 to TT8; each combination has four replicates under TT1; four replicates are labeled as (POT1A, POT1B, POT1C, and POT1D). Similarly, all treatments got their four replicates, including the control. Among eight combinations, TT1 was treated with *Trichoderma viride*; TT2 and TT3 with Bio-NPK; TT4 and TT5 with *T. viride* and Bio-NPK; TT6 and TT7 with Chemical NPK and TT8 was kept as the control. Dosage and concentration are depicted in Table 1; after transplanting in-vitro plants cultured into pots with sterile soil rite, the pots were inoculated with different concentrations of Bio-NPK, chemical NPK, and *T. viride* as a bio-control agent in some combinations. Initial data such as root length, number of roots, and shoot length were recorded at the time of transplantation. The irrigation was done periodically with sterile distilled water when required to maintain moisture. All recommended cultural practices, such as irrigation, removal of weeds, and plant protection, were adopted uniformly according to standard crop requirements. The first treatment of Bio-NPK was given in the first week on 7 April 2020, and the second was repeated in the first week of May on 7 May 2020. The bacterial population of the inoculants used as Bio NPK *B. megaterium*, *B. siamensis*, and *Azotobacter chroococcum* was 1.2 x 10^8 cells/cm^3. Bio-NPK was applied in two different concentrations, viz. 2.5 mL, 5 mL, and *Trichoderma* were used as bio-control. The CFU of *Trichoderma* used was $2x10^6$CFU/gram. Overall eight nutrient concentrations (mixed with *T. viride* and chemical NPK) were compared with each other and the control (without a non-inoculated pot) (Figure 3).

At 30, 60, and 90 days after transplanting the seedlings into the pots, the following attributes were used to measure. (A) Vegetative growth characters: Plant height, number of leaves and branches/plant, root biomass, and total plant biomass. (B) Yield components of tomato plants were calculated as, (i) Total Fruit weight (g)/pot, (ii) Total Fruit weight (g)/plant, (iii) Average fruit yield (g)/pot, (iv) Average fruit yield/plant (g), (v) Number and length of roots/plant (cm)

The result obtained are depicted in Tables 2, 3 for pot trial and Tables 4, 5, 6, and 7 for fields trial

Data analysis

The data values were compared with the control and chemically treated plants. All data are presented as the mean ± Standard Error (SE) of replicates and were analyzed using Data Processing Software (DPS, version 7.05) following one-way Analysis of Variance (ANOVA). Significant differences ($P < 0.05$) among treatment means after controlling for multiple comparisons were determined from a Least Significant Difference (LSD) test.

Tomato field trial experiment

Inside field conditions, Tomato field trial experiments were carried out inside GloBiL's campus field to verify the results determined from Tomato Pot Trial. The experimental field was cleared, harrowed, and sterilized. The field was divided into a set of four patches. In each set of four field patches, one patch was kept as control and labeled as TMC, two patches with 250 mL 500 mL Bio-NPK liquid, and the fourth patch with 250g chemical NPK (TM1-250 mL BIO-NPK, TM2-500mL BIO-NPK and TM3-250g chemical NPK) as depicted in Table 4. Tomato plant seedlings were procured from GloBiL's plantation department. Initial data were analyzed and recorded, like the height of plants, number of branches, root health, and shoot health. Irrigation was carried out whenever required. Bio-NPK liquid was applied in two patches, and chemical NPK in one patch. The control was without application, but control irrigation was done like other field patches. The bacterial population of the inoculants used as Bio NPK *(B. megaterium*, *B. siamensis*, and *Azotobacter)* was 1.2 x 10^8 cells/cm^3. Ten randomly selected plants from each patch at 30, 60, and 90 days from seed sowing were used to measure the vegetative growth characteristics such as plant height, number of leaves and branches/plant, plant fresh weight, and plant dry weight (Table 5).

RESULTS AND DISCUSSION

Plant growth promotion by Bio-NPK

With a plant growth promotion test, an increase in vegetative growth of tomato plants was observed after 30 days of treatment with Bio-NPK. The shoot length, root length, fruit yield, fruit weight, and total biomass were observed. It revealed a significant difference between the control and treated pots. Statistical analysis has shown a significant difference concerning the control (Figure 4).

Bio-NPK as biocontrol

Apart from PGPR activity, Bio-NPK was found to have biocontrol activity against *Fusarium* wilt and *Verticillium* wilt of tomato. Wilt infections were found in the control and chemically treated pots, but there were no cases from Bio-NPK-treated pots. The results were verified by carrying out the in vitro anti-fusarium activity of BIO-NPK against fusarium. Bio-NPK expressed very effective anti-fusarium activity.

Bio-NPK provides resistance against blossom end rot of tomato

The rhizosphere of plant species has an extensive range of microbes, including Bacillus and Pseudomonas. That shows avid rhizosphere colonization and plays an important role in crop production and yield (Podile and Kishore 2007).

The productivity of plants depends on the environmental stress they encounter in their natural habitat. (Oerke and Dehne 2004). Research has been done on plant-microbe interactions and has mainly focussed on the PGPR- induced alterations in the plant phenotype (Ali et al. 2011). This study aims to analyze the effect of the PGPR, *A. chroococcum, B. megaterium, B. siamensis,* and *T. viride* on root multiplication, growth, yield, biomass, and resistance to biotic and abiotic stress. All the Bio-NPK treated pots were found to be free from blossom end rot disease, while the control and chemically treated pots got blossom end rot disease. Hence it was concluded that Bio-NPK provides resistance against Blossom end rot of tomatoes (Figure 5).

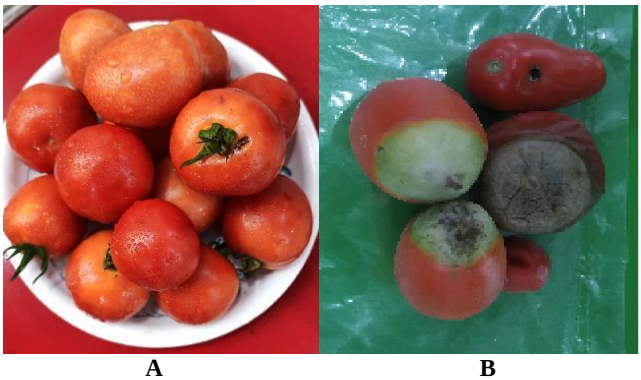
Bio-NPK and *Trichoderma* in plant growth promotion

After carrying out PGPR characterization tests, Bio-NPK was found to possess the capability to produce auxins, siderophores, and other compounds. That was supported by forming biofilms, phosphate and potassium solubilization, and nitrogen fixation. Furthermore, the plant growth promotion test performed in a greenhouse in pots showed the positive effect of Bio-NPK and *Trichoderma* on the enhancement of the growth of Tomato plants.

Figure 3. Pot trial of tomato plants

A B

Figure 4. Effect of Bio NPK on plant growth and root length. A. Untreated, B. Treated



A B

Figure 5. Resistance of plants against blossom end rots disease. A. Bio-NPK treated, B. Chemically treated

Data in (Tables 2 and 3) reveal that the total Biomass and fruit yield of Bio-NPK and *Trichoderma*-treated plant pots were significantly higher than untreated control or chemically treated pots. These results could be attributed to the synergistic effect of the microbial consortium. These results align with Barakat and Gabar (1998), who found that inoculation of tomato transplants with *A. Chroococcum, Azospirullum sps,* and *Bacillus polymyxa,* as single or mixed biofertilizers significantly increased the growth characteristics.

Bio-NPK and *Trichoderma* help in the biological control of diseases in tomato

The disease rate and severity of root rot and blossom end rot were the highest in untreated pots, and no cases were found in Bio-NPK and *Trichoderma* treated pots. However, the control without fertilizers and the control with chemical NPK were infected with blossom end rot (Figure 6). The *A. chroococcum* combined with *B. megaterium* and *B. siamensis* significantly controlled tomato plants' disease rate and severity compared to other treatments. These results are in harmony with those reported by Gupta et al. (1995), who found the combination of *A. chrocooccum* and biological agents (*T. harzanium and B. subtilis*) significantly decrease severity compared to the individual ones. The application of the biocontrol agent *T. harzanium* could control damping off and root rot disease of plants and enhance their survival rate (Niknejad et al. 2000)

Bio-NPK and *Trichoderma* enhance the quantity and quality of tomato yield

Data in (Table 2) indicated that yield components of tomato plants, i.e. number of fruits, the weight, fruits yield per plant, and size and color of the fruit, were the best in Bio-NPK treated plants as compared to the control (chemically treated and untreated or individually treated). Consortium inoculation (*B. megaterium, B. siamensis, Azotobacter,* and *Trichoderma*) significantly increased plants' yield and their components more than individual inoculation and chemically treated ones.

Fruit weight/pot

The effect of biofertilizers on average fruit weight was significant. The mean values showed that minimum and maximum average fruit numbers were observed in the first inflorescence in the control (311.625 ± 87.85) and BIO-NPK (748.925 ± 87.93), respectively. Table 2 shows the highest yield values with the quality and quantity of tomato plants observed in the inoculation treatment with BIO-NPK (*A. chroococcum, B. megaterium,* and *B. siamensis)* and *T. viride*. That is due to the synergistic effect of Bio-NPK and *T. viride.*

Martinez et al. (1994) and Radzi and Hisyamuddin. 2021 have found plants treated with biofertilizer containing *A. chroococcum, A. lipoferum,* and NPK results in the better crop in terms of yield and weight. In addition, these inoculants could also result in the early bloom of flowers as compared with the plants treated with nitrogen application only.

Bio-NPK and *Trichoderma* initiate the number and length of roots

Application of BIO-NPK increased plant yield, and mean value comparison was shown that minimum and maximum plant number and length of roots were in control (19.75 ± 1.31, 6.25 ± 0.75) and Bio-NPK (27 ± 0.57, 9 ± 0.20). The number and length of roots in Bio-NPK *Trichoderma* treated pots were more than individually treated pots and untreated pots (Tables 2 and 3; Figure 7).

Environmental stresses and a decline in soil health are becoming a major concern in declining productivity to a highly concerned level. Applying more chemical fertilizers and pesticides has further declined soil health and productivity. Biofertilizers are the solution at this moment when productivity is decreasing, and soil health is declining day by day. Biofertilizers provide a solution for sustainable agriculture with more productivity and soil health. The data obtained from the pot trial was analyzed in field conditions where two concentrations of Bio-NPK in liquid, amounting to 250 mL and 500 mL, were applied. In addition, the treatment was compared with the control and chemically NPK. The result is depicted in Tables 4, 5, 6 and 7.

Fruit number and weights

The effect of biofertilizers on average fruit number in field conditions was significant. The mean values showed minimum and maximum average fruit numbers in the first inflorescence in the control (TMC, 654 ± 14.62) and Bio-NPK(TM2, 810 ± 28.944), respectively (Table 6). The highest values of yield with quality and quantity of tomato plants were observed in the inoculation treatment with Bio-NPK (*A. chroococcum, B. megaterium,* and *B. siamensis)* at 500 mL concentration. The effect of liquid bio-fertilizer as Bio-NPK was significant in total fruit weight. The patch inoculated with 500mL BIO-NPK showed maximum fruit weight/patch (22917 ± 2869.72) compared to the control (11140 ± 1029.64) shown in Table 7.

There was an increase in total biomass in the chemically treated patch compared to liquid BIO-NPK and the control. However, the chemically treated patch and the control showed Blossom end rot disease. That is due to the synergetic effect of excess nitrogen (Taylor et al. 2004). In addition to imparting tolerance to several abiotic stress such as drought, salinity, and so on, *Trichoderma* seed treatment also ameliorates physiological stress such as aging and seed dormancy (Delgado-Sanchez et al. 2010, 2011)

The application of microorganisms as bio-fertilizers is a promising approach to assist in agricultural production; these applications have contributed to the growth of several crop species (Xiao et at. 2013) and increased plant biomass and total P contents (Jain et al. 2012). Bio-fertilizers keep the soil environment rich in all macro and micronutrients via nitrogen fixation; phosphate and potassium solubilization or mineralization; the release of plant growth regulating substances; production of antibiotics, and biodegradation of organic matter in the soil (Sinha et al. 2014).

Figure 6. Disease rate and disease severity of root rot, Blossom end rot. A: control, B, C, D: Chemical,NPK treated pots, E: Bio-NPK and *Trichoderma* treated pot

Figure 7. Comparison of root length of plants using different treatments

Table 1. Treatment information pot trial

Pot	TT1 POT A-D		TT2 POT A-D		TT3 POT A-D		TT4 POT A-D		TT5 POT A-D		TT6 POT A-D		TT7 POT A-D		TT8 POT A-D	
	TV	Bio-NPK	TV	Bio-NPK	TV	Bio-NPK	TV	Bio-NPK	TV	Bio-NPK	TV	Chemical-NPK	TV	Chemical-NPK	TV	Bio-NPK/chemical NPK

Note: -: indicate no treatment was given. TV: *Trichoderma viride*

Table 2. The yield of tomato plants grown under different fertilization treatments

Treatment	Total fruit weight (g)/plant	Total fruit weight (g)/pot	No. of roots/plant before treatment	No. of roots/plant after treatment
TT1	174.5 ± 21.01	349 ± 42.02	12.875 ± 1.06	22.5 ± 0.86
TT2	321.8125 ± 26.38	643.625 ± 52.76	13.25 ± 0.66	25.5 ± 0.86
TT3	374.4625 ± 43.96	748.925 ± 87.93	15 ± 0.97	27 ± 0.57
TT4	265.5 ± 43.34	531 ± 86.68	12.5 ± 1.17	24.5 ± 1.19
TT5	335.1375 ± 11.87	670.275 ± 23.75	16.625 ± 1.54	26.25 ± 0.47
TT6	236.25 ± 57.75	472.5 ± 115.50	9.25 ± 1.19	22.75 ± 1.25
TT7	263.5625 ± 41.84	527.125 ± 83.68	13 ± 2.35	24.25 ± 0.47
TT8	155.8125 ± 43.92	311.625 ± 87.85	11 ± 0.88	19.75 ± 1.31

Note: Data are means ± SE. Statistically significant differences between treatments ($P<0.05$)

Table 3. The root length, root biomass, and total plant biomass of tomato plants grown under different fertilization treatments

Treatment	Main root length/plant (cm) before treatment	Main root length/plant (cm) after treatment	Root biomass/plant	Total biomass/ treatment
TT1	1.825 ± 0.14	7.575 ± 0.64	174.4 ± 1.61	1626 ± 35.42
TT2	2.05 ± 0.18	8.375 ± 0.23	107.875 ± 0.65	2290 ± 54.94
TT3	1.875 ± 0.13	9 ± 0.20	134.125 ± 1.39	2568 ± 45.91
TT4	1.425 ± 0.19	8.375 ± 0.23	144.875 ± 3.35	2258 ± 15.19
TT5	1.85 ± 0.11	8.45 ± 0.47	133.25 ± 1.37	2522 ± 77.52
TT6	1.725 ± 0.04	7.75 ± 0.77	153.375 ± 0.89	2065 ± 36.42
TT7	1.575 ± 0.04	7.75 ± 0.47	137.125 ± 1.08	2251 ± 23.89
TT8	1.475 ± 0.06	6.25 ± 0.75	184.5 ± 25.38	1468 ± 20.44

Note: Data are means ± SE. Statistically significant differences between treatments (P<0.05)

Table 4. Treatment information field trial

Characters	TAG NAME	TM-C	TM-1	TM-2	TM3
Treatment/ concentration	BNPK-L	-	250ml	500ml	-
	NPK-C	-	-	-	250g
	WATER	500ml	500ml	500ml	500ml
No.of plants		20	20	20	20

Note: -: indicate no treatment was given. BNPKL: Bio-NPK liquid, NPK-C: Chemical NPK

Table 5. The height, number of branches, and leaf number of tomato plants grown under different fertilization treatments

Treatment	Plant height (cm) (DAT)			Number of leaves/plant (DAT)			Number of branches/plant (DAT)		
	30d	60d	90d	30d	60d	90d	30d	60d	90d
TMC	23.9 ± 1.15	28.5 ± 1.318	41.8 ± 2.85	24 ± 0.39	35.5 ± 1.10	42.3 ± 2.96	2.9 ± 0.23	4.4 ± 0.37	5.6 ± 0.37
TM-1	24.4 ± 1.06	32.1 ± 1.187	49 ± 1.17	24 ± 0.85	36 ± 1.06	49 ± 1.17	3.7 ± 0.3	5 ± 0.53	6.2 ± 0.24
TM-2	27.35 ± 0.93	33.85 ± 1.080	49.7 ± 1.15	26.3 ± 0.80	38.5 ± 0.94	49.7 ± 1.15	3.8 ± 0.29	5.5 ± 0.40	6.7 ± 0.36
TM3	27.1 ± 0.80	32.3 ± 1.258	48.8 ± 0.90	26.1 ± 0.50	40.3 ± 1.04	48.8 ± 0.90	4.3 ± 0.36	6.2 ± 0.44	7.1 ± 0.31

Note: Data are means ± SE. Statistically significant differences between treatments (P<0.05)

Table 6. The yield of tomato plants grown under different fertilization treatments

Treatment	No fruits/plant	No fruits/patch	Biomass/plant(g)	Biomass/patch(g)
TMC	32.7 ± 0.73	654 ± 14.62	217.1 ± 18.17	4342 ± 363.40
TM-1	33.5 ± 1.07	670 ± 21.55	294.1 ± 28.84	5800.2 ± 1010.12
TM-2	40.5 ± 1.44	810 ± 28.944	337 ± 33.20	5882 ± 576.85
TM3	38.4 ± 1.51	768 ± 30.28	360.6 ± 45.53	8017 ± 377.19

Note: Data are means ± SE. Statistically significant differences between treatments (P<0.05)

Table 7. The yield of tomato plants grown under different fertilization treatments

Treatment	Fruit weight/plant (g)	Fruit weight/patch (g)
TMC	557 ± 51.48	11140 ± 1029.64
TM-1	813.77 ± 178.11	16275.4 ± 3562.30
TM-2	1145.85 ± 143.48	22917 ± 2869.72
TM3	962.697 ± 176.51	19253.94 ± 3530.24

Note: Data are means ± SE. Statistically significant differences between treatments (P<0.05)

Long-term chemical fertilizer usage greatly reduced bacterial colonies and their richness in brown soil (Tang et al. 2021). The added inputs of fertilizers accumulate with time and result in environmental pollution, causing problems to human and animal health (Alori and Bababola 2017). Moreover, chemical fertilizer significantly decreased the Chao and ACE richness indexes of the bacterial community but increased the fungal community (Wang et at. 2017).

Moreover, using this study method on tomato plants with Bio-NPK and *Trichoderma* interaction, the bacterial consortium's positive impact on Tomato growth, yield, disease resistance, and biocontrol could be reported. Activation of multiple defense signals by Bio-NPK and

Trichoderma proves their role in plant defense and as a biocontrol agent. Further, the Bio-NPK and *Trichoderma* impact on an increase in overall biomass and product yield proves their role in plant growth promotion. With all these data, we can conclude that PGPR influences plants' defense mechanisms and confer plants' immunity to protect themselves against attack by phytopathogens.

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REFERENCES

Ali SK, Sandhya V, Grover M, Rao LV, Venkateswarlu B 2011. Effect of inoculation with a thermotolerant plant growth promoting *Pseudomonas putida* strain AKMP7 on the growth of wheat (*Triticum* spp.) under heat stress. J Plant Interact 6: 239-246. DOI: 10.1080/17429145.2010.545147.

Alori ET, Bababola OO. 2017. Microbial inoculants for improving crop quality and human health in Africa. Front Microbiol 9: 2213. DOI: 10.3389/fmicb.2018.02213.

Bais HP, Park SW, Weir TL, Callaway RM, Vivanco JM. 2004. How plants communicate using the underground information superhighway. Trends Plant Sci 9 (1): 26-32. DOI: 10.1016/j.tplants.2003.11.008.

Barakat MAS, Gabar SM. 1998. Effect of different biofertilizer types and nitrogen fertilizer levels on tomato plants. Alexandria. J Agric Res 43 (1): 149-160.

Barnawal D, Singh R, Singh RP. 2019. Role of Plant Growth Promoting *Rhizobacteria* in Drought Tolerance: Regulating Growth Hormones and Osmolytes. PGPR Amelioration in Sustainable Agriculture. DOI: 10.3390/agronomy9110712.

Bhat MA, Kumar V, Bhat MA, Wani IA, Dar FL, Farooq I, Bhatti F, Koser R, Rahman S, Jan AT. 2020. Mechanistic insights of the interaction of Plant Growth Promoting Rhizobacteria (PGPR) with plant roots toward enhancing plant productivity by alleviating salinity stress. Front Microbiol 11: 1952. DOI: 10.3389/fmicb.2020.01952.

Blackshaw RE. 2005. Nitrogen fertilizer, manure, and compost effects on weed growth and competition with spring wheat. Agronomy J 97 (6): 1612-1621. DOI: 10.2134/agronj2005.0155.

Bric JM, Bostock RM, Silverstone SE. 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 57: 535-538. DOI: 10.1128/aem.57.2.535-538.1991.

Chawngthu L, Hnamte R, Lalfakzuala R. 2020. Isolation and characterization of rhizospheric phosphate solubilizing bacteria from wetland paddy field of Mizoram, India. Geomicrobiol J 37 (4): 366-375. DOI: 10.1080/01490451.2019.1709108.

Delgado-Sanchez P, Ortega-Amaro MA, Jimenez-Bremont JF, Flores J. 2011. Are fungi important for breaking seed dormancy in desert species? Experimental evidence in *Opuntia streptacantha* (Cactaceae). Plant Biol (Stuttg) 13 (1): 154-9. DOI: 10.1111/j.1438-8677.2010.00333.x.

Dutta S, Podile AR. 2010. Plant Growth Promoting *Rhizobacteria* (PGPR): The bugs to debug the root zone. Crit Rev Microbiol 36 (3): 232-244. DOI: 10.3109/10408411003766806.

Fu L, Penton CR, Ruan Y, Shen Z, Xue C, Li R, Shen Q. 2017. Inducing the rhizosphere microbiome by biofertilizer application to suppress banana Fusarium wilt disease. Soil Biol Biochem 104: 39-48. DOI: 10.1016/j.soilbio.2016.10.008.

Gupta R, Sharma JP. 2018. Characterization of PSB isolates in Rhizosphere soil of rice varieties grown at OFRC, SKUAST Jammu. Intl J Curr Microbiol Appl Sci 7 (09): 1-8. DOI: 10.20546/ijcmas.2018.709.001

Gupta S, Arora DK, Srivastava AK. 1995. Growth promotion of tomato plants by rhizobacteria and imposition of energy stress on *Rhizoctonia solani*. Soil Biol Biochem 27 (8): 1051-1058. DOI: 10.1016/0038-0717(95)00011-3.

Jain R, Saxena J, Sharma V. 2012. Effect of phosphate solubilizing fungi *Aspergillus awamori* S29 on mungbean (*Vigna radiata* cv. RMG 492) growth (533-541). Folia Microbiol (Praha) 57 (6): 533-41. DOI: 10.1007/s12223-012-0167-9.

Khan N, Ali S, Tariq H, Latif S, Yasmin H, Mehmood A, Shahid MA. 2020. Water conservation and plant survival strategies of rhizobacteria under drought stress. Agronomy 10 (11): 1683. DOI: 10.3390/agronomy10111683.

Khanna K, Jamwal VL, Kohli SK, Gandhi SG, Ohri P, Bhardwaj R, Wijaya L, Alyemeni MN, Ahmad P. 2019a. Role of Plant Growth Promoting Bacteria (PGPRs) as biocontrol agents of Meloidogyne incognita through improved plant defense of *Lycopersicon esculentum*. Plant Soil 436 (1-2): 325-345. DOI: 10.1007/s11104-019-03932-2.

Khanna K, Jamwal VL, Sharma A, Gandhi SG, Ohri P, Bhardwaj R, Al-Huqail AA, Siddiqui MH, Ali HM, Ahmad P. 2019b. Supplementation with Plant Growth Promoting *Rhizobacteria* (PGPR) alleviates cadmium toxicity in *Solanum lycopersicum* by modulating the expression of secondary metabolites. Chemosphere 230: 628-639. DOI: 10.1016/j.chemosphere.2019.05.072.

Khanna K, Kohli SK, Ohri P, Bhardwaj R, Alhuqail AA, Siddiqui MH, Alosaimi GS, Ahmad P. 2019. Microbial fortification improved photosynthetic efficiency and secondary metabolism in *Lycopersicon esculentum* plants under Cd stress. Biomolecules 9: 581. DOI: 10.3390/biom9100581.

Lalitha S. 2017. Plant growth promoting microbes: A boon for sustainable agriculture sustainable. In: Dhanarajan A (eds). Sustainable Agriculture towards Food Security. Springer Nature Singapore Pte Ltd., Singapore. DOI: 10.1007/978-981-10-6647-4_8.

Martinez VR, Dibut AB, Gonzalez PR, Acosta RM. 1994. Effect of the application of biopreparations based on Azotobacter chroococcum on tomato and onion crops in red ferrallitic soils. 90-anos-de-la-Estacion Experimental-Agronomica-de-Santiago-de-lasVegas. 167-184. (C.F. CAB Abstracts computer research 1996-1998).

Mehnaz S, Baig DN, Lazarovits G. 2010. Genetic and phenotypic diversity of plant growth promoting rhizobacteria isolated from sugarcane plants growing in Pakistan. J Microbiol Biotechnol 20: 1614-1623. DOI: 10.4014/jmb.1005.05014.

Meyer JM, Abdallah MA. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. Microbiol 107: 319-328. DOI: 10.1099/00221287-107-2-319.

Morikawa M, Kagihiro S, Haruki M, Takano K, Branda S, Kolter R, Kanaya S. 2006. Biofilm formation by a *Bacillus subtilis* strain that produces c-polyglutamate. Microbiol 152: 2801-2807. DOI: 10.1099/mic.0.29060-0.

Muhammad S, Arshad M, Hussain S, Bhatti AS. 2007. Perspective of Plant Growth Promoting *Rhizobacteria* (PGPR) containing ACC deaminase in stress agriculture. J Indus Microbiol Biotechnol 34 (10): 635-648. DOI: 10.1007/s10295-007-0240-6.

Nautiyal CS. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170: 265-270. DOI: 10.1111/j.1574-6968.1999.tb13383.x.

Niknejad M, Tehani AS, Okhovat M. 2000. effect of antagonistic fungi *Trichoderma* spp. on the control of *Fusarium* wilt of tomato caused Fusarium oxysporum f.sp. lycopersici under greenhouse conditions. Iranian J Agric Sci 1: 31-37. DOI: 10.1007/s11033-019-04901-9.

Oerke EC, Dehne HW. 2004. Safeguarding production losses in major crops and the role of crop protection. Crop Prot 23: 275-285. DOI: 10.1016/j.cropro.2003.10.001.

Ortíz-Castro Randy, Contreras-Cornejo HA, Macías-Rodríguez L, López-Bucio J. 2009. The role of microbial signals in plant growth and development. Plant Signal Behav 4 (8): 701-712. DOI: 10.4161/psb.4.8.9047.

Parray JA, Jan S, Kamili AN, Qadri RA, Egamberdieva D, Ahmad P. 2016. Current perspectives on plant growth-promoting rhizobacteria. J Plant Growth Regul 35 (3): 877-902. DOI: 10.1007/s00344-016-9583-4.

Pérez-Montaño F, Alías Villegas C, Bellogín RA, del Cerro P, Espuny MR, Jiménez Guerrero I, López Baena FJ, Ollero FJ, Cubo T. 2014. Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. Microbiol Res 169 (5-6): 325-336. DOI: 10.1016/j.micres.2013.09.011.

Podile AP, Kishore GA 2007. Plant Associated Bacteria. Kluwer Academic Publishers, Dordrecht. DOI: 10.1007/1-4020- 4538-7_6.

Qiao J, Yu X, Liang X, Liu Y, Borriss R, Liu Y. 2017. Addition of plant growth promoting *Bacillus subtilis* PTS-394 on tomato rhizosphere has no durable impact on composition of root microbiome. BMC Microbiol 17: 1-12. DOI: 10.1186/s12866-017-1039-x.

Qiu M, Zhang R, Xue C, Zhang S, Li S, Zhang N, Shen Q. 2012. Application of bio-organic fertilizer can control *Fusarium* wilt of cucumber plants by regulating microbial community of rhizosphere soil. Biol Fertil Soils 48: 807-816. DOI: 10.1007/s00374-012-0675-4.

Radzi NM, Hisyamuddin MRA. 2021. The effect of different rate of biofertilizer on the growth performance and yield of rice. IOP Conf Ser Earth Environ Sci 757 (1): 012050. DOI: 10.1088/1755-1315/757/1/012050.

Sinha RK, Valani D, Chauhan K, Agarwal S. 2014. Embarking on a second green revolution for sustainable agriculture by vermiculture biotechnology using earthworms: reviving the dreams of Sir Charles Darwin. Intl J Agric Health Saf 1: 50-64.

Tang H, Li C, Shi L, Xiao X, Cheng K, Wen L, et al. 2021. Effect of different long-term fertilizer managements on soil nitrogen fixing bacteria community in a double-cropping rice paddy field of southern China. PLoS ONE 16 (9): e0256754. DOI: 10.1371/journal.pone.0256754

Teng F, Nair SSD, Zhu P, Li S, Huang S, Li X, Xu J, Yang F. 2018. Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. Sci Rep 8: 1-12. DOI: 10.1038/s41598-018-34294-x.

Wang Z, Li T, Wen X, Lui Y, Han J, Liao Y, DeBruyn JM. 2017. Fungal communities in Rhizosphere soil under conservation tillage shift in response to plant growth. Front Microbiol 8: 1-11. DOI: 10.3389/fmicb.2017.0130.

Wani SP, Rupela OP, Lee KK. 1995. Sustainable agriculture in the semi arid tropics through biological nitrogen fixation in grain legumes. Plant Soil 174: 29-49. DOI: 10.1007/bf00032240.

Widawati S, Suliasih. 2006. The population of Phosphate Solubilizing Bacteria (PSB) from Cikaniki, Botol Mountain, and Ciptarasa Area, and the ability of PSB to solubilize insoluble P in solid pikovskaya medium. Biodiversitas 7 (2): 109-113. DOI: 10.13057/biodiv/d070203.

Xiao C, Zhang H, Fang Y. et al. 2013. Evaluation for Rock Phosphate Solubilization in Fermentation and Soil–Plant System Using A Stress-Tolerant Phosphate-Solubilizing Aspergillus niger WHAK1. Appl Biochem Biotechnol 169: 123-133. DOI: 10.1007/s12010-012-9967-2

Yaseen R, Aziz O, Saleem MH, Riaz M, Zafar-ul-Hye M, Rehman M, Ali S, Rizwan M, Alyemeni MN, El-Serehy HA, Al-Misned FA, Ahmad P. 2020. Ameliorating the drought stress for wheat growth through application of ACC-Deaminase containing *Rhizobacteria* along with Biogas Slurry. Sustainability 12 (15): 6022. DOI: 10.3390/su12156022.

Zhang J, Zhu T, Cai Z, Qin S, Müller C. 2012. Effects of long-term repeated mineral and organic fertilizer applications on soil nitrogen transformations. Eur J Soil Sci 63: 75-85. DOI: 10.1111/j.1365-2389.2011.01410.x.

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Green synthesis of copper nanoparticles using *Musa acuminata* aqueous extract and their antibacterial activity

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Abstract. *Muhammad A, Umar A, Birnin-Yauri AU, Sanni HA, Elinge CM, Ige AR, Ambursa MM. 2023. Green synthesis of copper nanoparticles using* Musa acuminata *aqueous extract and their antibacterial activity. Asian J Trop Biotechnol 20: 10-16.* Green synthesis is a convenient and affordable alternative to the synthesis of copper nanoparticles. The objective of the present study was to synthesize copper nanoparticles via the green method, using *Musa acuminata* (banana) fruit extracts as a reducing agent with copper sulfate serving as a precursor. The antibacterial activities of the synthesized CuNps were also tested. Fruits extracts of *M. acuminata* were used to synthesize with $CuSO_4$(aq) as a precursor. The synthesized nanoparticles were characterized using UV-Vis, FTIR, XRD, SEM, and EDX techniques. The synthesis results showed that the nanoparticles were of Face-Centered Cubic (FCC) structure with high stability. The average diameter of the synthesized copper nanoparticles is 18nm. UV-Vis, scanning electron microscope, X-ray diffraction, energy disperses X-ray, and Fourier transforms infrared spectroscopy established that the formed nanoparticles are copper nanoparticles. The antibacterial activity of the synthesized nanoparticles was also tested using pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. The results showed that copper nanoparticles were promising antibacterial agents.

Keywords: Antibacterial activity, banana, copper nanoparticles, $CuSO_4$ solution, *Musa acuminata* extract

INTRODUCTION

Green synthesis can be defined as the derivation of materials from green or eco-friendly resources by using solvents, excellent reducing agents, and harm less material for stabilization (Bonde 2011: Sable et al. 2012; Jadoun et al. 2021). Furthermore, this synthesis route is simple, economical, reliable, sustainable, and relatively reproducible and provides more stable compounds. Therefore, researchers are interested in developing various nanomaterials through this biosynthesis pathway, including metal/metal oxide nanoparticles, hybrid materials, and biologically inspired materials. As a result, green synthesis is widely regarded as a necessary tool to reduce the negative effects of conventional nanoparticle synthesis methods used in laboratories and industry (Yedurkar et al. 2017).

With numerous reports and studies, this green synthesis process already produces a large number of metal/metal oxide nanoparticles such as silver (Ag), gold (Au), selenium (Se), platinum (Pt), zinc oxide, etc. (Chakraborty et al. 2022). Furthermore, the studies also reported that some metallic nanoparticles have a variety of biological and biochemical activities, but CuNPs have recently received a lot of attention. Copper plays a variety of roles in humans health, including the production of neuropeptides, regulation of cell signaling pathways, antioxidant defense, and co-factoring of many enzymes involved in immune cell function (Georgopoulos et al. 2001; Ghaderian and Ravandi 2012).

Copper Nanoparticles (CuNPs) attract positive global attention because of their low-cost and novel optical, mechanical, catalytic, electrical and thermal conduction properties, which differ from that of bulk metals (Brust and Kiely 2002). Copper nanoparticles have been considered alternatives for noble metals in many applications, such as heat transfer and microelectronics. However, the synthesis of copper nanoparticles poses a challenge due to the high tendency for oxidation. Unlike gold and silver, copper is extremely sensitive to air, and the oxide phases are thermodynamically stable (Jeong et al. 2008).

Plants produce many biologically active chemicals. Because very small amounts of these heavy metals are hazardous even at very low concentrations, plants have shown extraordinary potential in detoxifying and accumulating heavy metals, which may allow them to outrun environmental pollutants (Chakraborty et al. 2022). In addition, the synthesis of nanoparticles using plant extracts is superior to other biological synthesis methods, such as microorganisms. That is because the rate of synthesis of metallic nanoparticles using plant extracts is more sustained (Saif et al. 2016), significantly faster and more monodisperse than other biological methods (Sarkar et al. 2020).

Copper nanoparticles have been synthesized using extracts from various plants found all over the globe (Murthy et al. 2020). From this note, many plant parts, fruits, or whole plants have been used for the green

synthesis of CuNPs due to the presence of a large number of bioactive compounds (Murthy et al. 2020). The Synthesis of CuNPs has been successfully extracted from various parts of *Punica granatum* (Kaur et al. 2016), *Zingiber officinale* stem (Delma and Rajan 2016), *Citrus medica* juice (Shende et al. 2015), *Ziziphus spina-christi* fruit (Khani et al. 2018), *Asparagus adscendens* roots and leaves (Thakur et al. 2018); leaves of *Eclipta prostrata* leaves (Chung et al. 2017); *Ginkgo biloba* leaves (Nasrollahzadeh and Mohammad 2015); *Plantago asiatica* leaves (Nasrollahzadeh et al. 2017); *Thymus vulgaris* (Issaabadi et al. 2017); Black tea leaves (Asghar et al. 2018); *Terminalia catappa* leaves (Muthulakshmi et al. 2017) and *Azadirachta indica* (Ahmed et al. 2015).

This research work synthesized CuNP from copper sulfate pentahydrate and aqueous extract from *Musa acuminata*. Advanced techniques characterized the copper nanoparticles, and an antimicrobial assay was performed.

MATERIALS AND METHODS

The fruits of *M. acuminata* (banana) were obtained from Sokoto central market for this study. The representative samples for the analysis were selected by random sampling. The samples of the banana fruits were peeled, and 20 g were accurately weighed. The sample was then crushed and finely macerated with mortar and pestle. The sample was then added to 100cm^3 of deionized water and boiled over a water bath at 60°C for 15 minutes. The extract was allowed to cool down and filtered with muslin cloth through Whatman filter paper and used immediately for the synthesis. A 0.01 M solution of $CuSO_4.5H_2O$ was prepared by dissolving 2.4955 g of the salt in 10 cm^3 of deionized water.

Green synthesis of copper nanoparticles

About 10 cm^3 of the *M. acuminata* extract was added to 100 cm^3 of 0.01 M $CuSO_4.5H_2O$ aqueous solution and mixed thoroughly. The mixture was heated to 80°C with constant stirring on a magnetic stirrer for six h. The suspension produced was centrifuged at 3,000 rpm for 10 min, and the supernatant liquid was decanted. The residue was repeatedly washed with 10 cm^3 of deionized water. Centrifugation-decantation-washing processes were repeated five times to remove impurities on the surface of the copper nanoparticles. The obtained precipitates were dried in an oven at 50°C for 24 h. The synthesized copper nanoparticles were then kept for characterization and antimicrobial studies.

Characterization

UV-Visible spectroscopy

UV-Visible spectroscopy technique was used in this research to confirm the formation of nanoparticles. For each analysis, 1.0 cm^3 of the aliquot suspension was diluted in 4.0 cm^3 deionized water, and its UV-Visible spectrum was measured at 80°C (Gnanasangeetha and Prathipa 2019).

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was carried out to identify the possible biomolecules responsible for reducing copper sulfate to CuNPs and the capping and stabilizing agents for CuNPs. After synthesizing the copper nanoparticles, the precipitate obtained using each extract was dried in an oven at 50°C for 24 h. The dried synthesized copper nanoparticles were grounded with KBr, cast into a pellet, and used for analysis on the FTIR spectrophotometer in the diffuse reflectance mode operating at a resolution of 4 cm^{-1} (Gnanasangeetha and Sarala 2015).

X-Ray Diffraction studies (XRD)

X-ray diffraction was used to determine the phase and crystallinity of the nanoparticles. The dried samples of the as-synthesized copper nanoparticles were grounded to fine powder and a thin film of each sample was made by dipping a cleaned glass plate into the powdered nanoparticles of the sample. The XRD analysis was performed passing monochromatic Cu radiation (θ = 1.5406 Å) operating at a voltage of 40kV and a current of 25mA on the sample film at room temperature (25°C). The XRD patterns were then collected at 2θ angles between 10° and 80°, 0.02 min$^{-1,}$ and at 1 second time constant. The peaks obtained were compared with the reference database in the Joint Committee on Powder Diffraction Standards (card no: 53-61386) library to ascertain the nature of the nanoparticles (Gnanasangeetha and Sarala 2015).

Scanning Electron Microscopy (SEM)

The SEM is a multipurpose instrument that provides qualitative information about the samples, such as topography, morphology, composition, and crystallographic arrangements, with high resolution (Gnanasangeetha and Suresh 2020). First, the surface morphology of the synthesized copper nanoparticles was studied using Scanning Electron Microscopy (SEM). Next, the sample was prepared to study size and crystallographic structure by placing a drop of colloidal solution of the synthesized copper nanoparticles in copper (II) sulfate on a carbon-coated copper slide and subsequently drying it in the air before transferring it to the microscope. The operation was carried out at an accelerated voltage of 130 kV, magnification of X^{10}, and resolution of 1 nm (Sarala 2015).

Energy Dispersive X-ray (EDX)

The use of EDX analysis investigated the presence of metallic copper. The copper nanoparticles' elemental composition, purity, and geometry were studied. Copper nanoparticles had a sorption peak at 1Kev (Ebrahimi et al. 2017), the point at which the index for metallic nanoparticles of copper was expected. The EDX observation was carried out by instrument coupled with SEM (Gnanasangeetha and Sarala 2015).

Test of antibacterial activity

The antibacterial activity of the synthesized copper nanoparticles was investigated according to the method outlined by Ebrahimi et al. (2017). The assays were

conducted on agar well diffusion method with Gram-negative bacteria *Escherichia coli* and Gram-positive bacteria *Staphylococcus aureus.* The pathogenic bacteria were cultured using nutrient agar in petri dishes with an inner diameter of 9 cm to provide a thin agar plate of thickness 3.4-3.5 mm after solidification. The culture was regulated to 0.5 McFarland standards to get 1.5×10^6 CFU/mL (Ebrahimi et al. 2017).

The sample was prepared for an antibacterial test and labeled as A for the banana (*M. acuminata*) mediated Cu nanoparticles. The extracts were prepared at 1 mg/mL and dissolved in Dimethyl Sulfoxide (DMSO) in each case (Murthy et al. 2020). A Hollow of 6mm diameter was cut from each assay using a sterile cork-borer, and 50 µL of each of the three extracts was impregnated using a sterilized wire loop on the surface of Mueller-Hinton Agar (MHA) plates. The pathogens were incubated at 5-8°C for 2 hours to ensure good diffusion and then further incubated at 37°C for 24 hours (Ebrahimi et al. 2017). Ampicillin disc was used as a control. Next, using a micropipette, 10µL of antibiotic control (Ampicillin) was measured and used for both the *E. coli* and the *S. aureus* bacteria (Ebrahimi et al. 2017). The diameters of the inhibition zone in each case were measured millimeters using a ruler, and the results were recorded.

RESULTS AND DISCUSSIONS

UV-Visible absorption spectroscopic study

UV-Visible spectra of aqueous $CuSO_4.5H_2O$ mediated with the *M. acuminata* fruits extract are shown in Figure 1. The reduction of Cu^{2+} ions to Cu nanoparticles by the sample extract was indicated by the change of color from pale yellow to reddish brown.

The progress in reducing Cu^{2+} ions to Cu nanoparticles was indicated by the enhanced intensity of surface Plasmon absorption peak observed within 560nm to 600nm. The maximum absorption observed was 574nm. The appearance of the peak assigned to the Surface Plasmon Resonance band is within the range of 550nm-600nm for copper nanoparticles, as reported by Khodaie and Ghasemi (2018).

The metal nanoparticles show strong absorption of visible radiation due to their induced polarization in their conduction electrons concerning the immobile nucleus (Michael 2012). Furthermore, when a particular wave length is matched to the size of a nanoparticle, dipole oscillation is generated in the compensated form of the induced polarization, and the electrons in the nanoparticle resonate, introducing a strong absorption (Moskovits and Vlckova 2005).

Fourier Transform Infrared spectroscopic study (FTIR)

FTIR spectroscopic measurement was conducted to discover the possible biomolecules in the *M. acuminata* (banana) fruit extract and the synthesized Cu nanoparticles. Figure 2 showed active functional groups in the fruit extracts and the functional groups in the synthesized CuNPs. The figures showed a comparative Fourier Transform Infrared (FTIR) spectroscopic analysis of sample extracts and the synthesized CuNPs.

Peaks observed around 3,200 cm^{-1} and 2,900 cm^{-1} are characteristics of a carboxylic COOH (or N-H stretching mode) and alkynic ≡C-H stretching. Peaks at 1,700 cm^{-1}, 1,600 $cm^{-1}$and 1,618 cm^{-1} are corresponding to amide, arising due to carbonyl stretching in proteins, and the band at 1,604 cm^{-1} is a characteristic of N-H bending. The peaks at 1,474 and 1,039-1,381cm^{-1} correspond to methylene scissoring vibrations from the proteins in the solution and C-N stretching vibrations of amine (Michael 2012). The FTIR spectra showed that the flavonoids, alkaloids, protein molecules, and other metabolites in the fruit extract are responsible for reducing copper ions and stabilizing the Cu nanoparticles. The FTIR data were in agreement with the previous reports (e.g., Dadgostar 2008; Hailemariam 2011; Valodkar et al. 2011; Kalainila et al. 2014; Gnanasangeetha and Prathipa 2019). It is also clear from the relative FTIR data that the peaks of the functional groups of the spectra of the fruit extract and those of their corresponding synthesized copper nanoparticles are almost similar. That signifies the impact of the functional groups in the fruit extracts in reducing Cu^{2+} to CuO and further stabilization of CuNPs, as stated by Chandraker et al. (2020).

X-Ray Diffraction studies (XRD)

The XRD pattern of the synthesized Cu nanoparticles obtained by the green reduction of copper ions using banana (*M. acuminata*) fruit extracts is presented in Figure 3. The values of experimental diffraction peaks observed in the patterns at 2θ for CuNPs synthesized from banana fruit extract are 43.50, 50.40, and 74.10. These values of diffraction data conformed with Inorganic Structure Data Base (ICSD); file no. 04-0836 (Rajesh et al. 2018) as well as with International Centre for Diffraction Data (ICDD) standard for value for CuNPs; file number 04-0836 (Theivasanthi and Alagar 2011). The three distinct diffraction peaks for the copper nanoparticles synthesized from the banana correspond to (111), (200), and (220) lattice planes of the face-centered cubic structure (FCC) of copper nanoparticles.

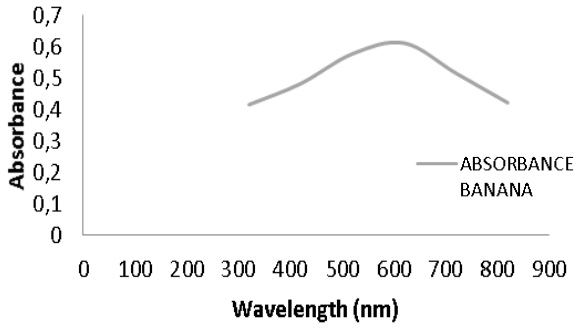

Figure 1. UV-Visible spectra of the synthesis of copper nanoparticles for the three fruits extracts

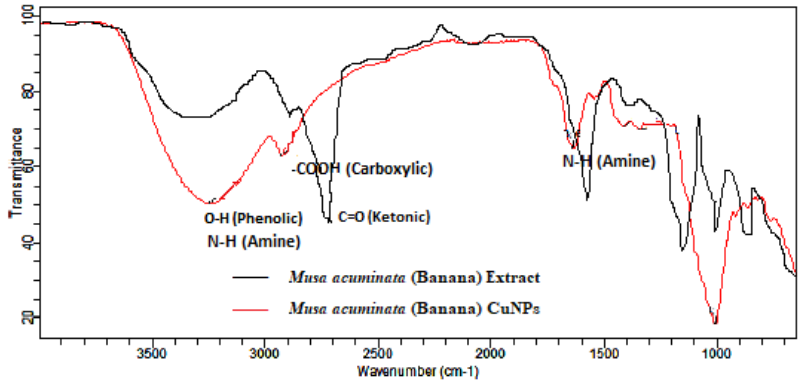

Figure 2. FTIR spectra of *Musa acuminata* (banana) extract and CuNPs synthesized using *M. acuminata* extract

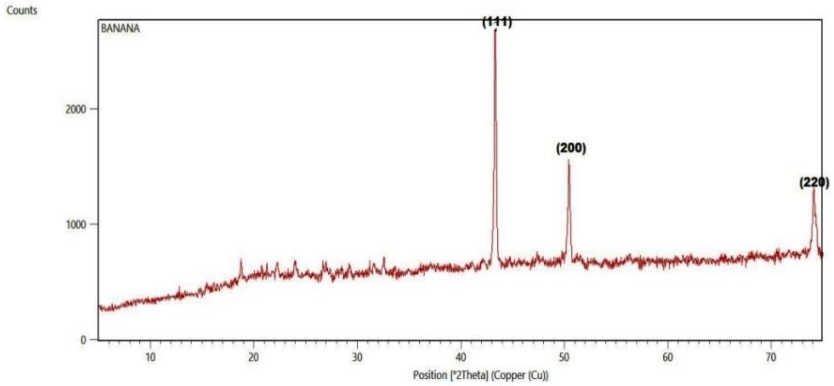

Figure 3. XRD Pattern of CuNPs synthesized using *Musa acuminata* (banana)

Scanning Electron Microscopy (SEM)

Figure 4 presents an image obtained from Scanning Electron Microscopy (SEM) of the copper nanoparticles synthesized from banana. Figure 4 presents SEM images of copper nanoparticles synthesized from bananas, which showed dispersed copper nanoparticles with irregular edges. The diameters of the copper nanoparticles were 18 nm for the banana, and the shapes of the CuNPs synthesized from banana synthesized nanoparticles appeared dispersed. The results were in agreement with Jaehoon et al. (2006) and Arya et al. (2018).

Energy Dispersive X-ray (EDX)

The use of EDX analysis confirmed the presence of metallic copper. The observed chart for Energy Dispersive X-ray (EDX) for as-synthesized copper nanoparticles is illustrated in Figure 5.

The EDX spectra of the copper nanoparticles synthesized are presented in Figure 5. The EDX spectra confirmed the presence of metallic copper and other elements, which corroborated the findings of Gnanasangeetha and Sarala (2015). The presence of elements such as C, S, and Cl may be attributed to contaminations originating from the biomolecules bound to the surface of the copper nanoparticles or physical absorption during sample preparation. In addition, oxygen and the Cu signal signify that phytoconstituents capped the CuNPs through oxygen atoms. In contrast, the presence of a trace amount of carbon demonstrated the involvement of plant phytochemical groups in the reduction and capping of the synthesized CuNPs.

Antibacterial activities of copper nanoparticles

The antibacterial activities of banana (*M. acuminata*) fruits extract mediated copper nanoparticles against two pathogenic bacteria, Gram-negative *E. coli* and Gram-positive *S. aureus* using agar well diffusion method shown in Table 1. Figure 6 illustrated the antibacterial activity of *M. acuminata* synthesized CuNps and Ampicillin, respectively, on *S. aureus* and *E. coli* bacteria.

The mean values of three replicates of the zone of inhibition in (mm) around each assay of the synthesized copper nanoparticles are presented in Table 1. The results of antibacterial activities of banana (*M. acuminata*) fruits extract mediated copper nanoparticles against two pathogenic bacteria, Gram-negative *E. coli* and Gram-positive *S. aureus* using agar well diffusion method and are presented in Table 1. The diameter of the assays' inhibition zones was measured in millimeters (mm) using a ruler. The results were recorded and compared to those obtained from the standard antibiotic drug, Ampicillin.

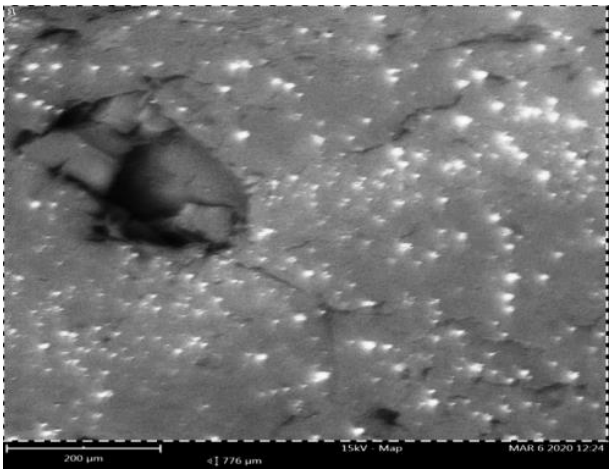

Figure 4. SEM image of CuNPs synthesized using *Musa acuminata* (banana)

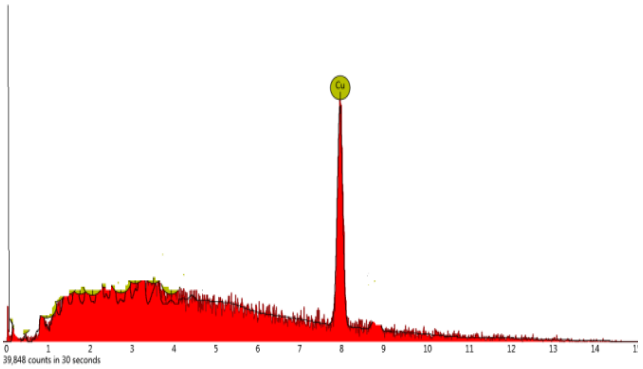

Figure 5. EDX figure of CuNPs synthesized using *Musa acuminata* (banana)

Figure 6. A. The antibacterial activity of *Musa acuminata* synthesized CuNps (A) and Ampicillin (B), respectively, on *S. aureus* and *E. coli* bacteria

Table 1. Mean values of three replicates of the zone of inhibition (mm) around the sample of the synthesized copper nanoparticles

Culture (bacteria)	Diameter of zone of inhibition (mm)	
	***Musa acuminata* (banana)**	**Ampicillin (Standard)**
Escherichia coli	16±2.2	17±0.50
Staphylococcus aureus	19±2.5	19±1.20

Note: The zone of inhibition was measured in mm. Values are expressed as Mean±SD, where n=3

From the obtained results, copper nanoparticles were found to have higher antibacterial activity against Gram-positive bacteria *S. aureus* than Gram-negative *E. coli*. The antibacterial activity was also more pronounceable against *S. aureus* compared to Ampicillin. The inhibition zones observed for *M. acuminata* synthesized CuNPs are 19±2.5 for *S. aureus* and 16±2.2 for *E. coli* and Ampicillin 19 mm and 17 mm are the values recorded for *S. aureus* and *E. coli*, respectively.

The higher activity against Gram-positive may be attributed to a greater abundance of amines and carboxyl groups on the cell surface of the Gram-Positive Bacteria and a greater affinity of copper towards these groups. According to Rupareli et al. (2008), copper ions released may interact with DNA molecules and insert between the nucleic acid strands. Moreover, copper ions inside bacterial cells also disrupt biochemical processes. The study showed that green synthesized copper nanoparticles from bananas have great potential in biomedical applications. That following with the previous observations of other researchers (Hailemariam 2011; Theivasanthi and Alagar 2011; Joseph et al. 2016).

In conclusion, this research confirmed the feasibility of green synthesis of copper nanoparticles using extracts of *M. acuminata* (banana) fruits. The results indicated a reduction of copper ions and stabilization of copper nanoparticles occurred due to the presence of proteins and other metabolites in the fruit extracts. The size of synthesized CuNPs calculated using powder XRD pattern is 18nm. FT-IR results confirmed that extracts of *M. acuminata* (banana) fruit are a suitable green pattern to

prepare copper nanoparticles. It was also observed that *M. acuminata* (banana) copper nanoparticles were found to have a very good and fabulous antibacterial activity.

REFERENCES

Ahmed S, Saifullah M, Ahmad B, Swami L, Ikram, S. 2015. Green synthesis of silver nanoparticles using *Azadirachta indica* aqueous leaf extract. J Radiat Res Appl Sci 9 (1): 1-7. DOI: 10.1016/j.jrras.2015.06.006.

Arya, A, Gupta, K, Chundawat, TS, Vaya D. 2018. Biogenic synthesis of copper and silver nanoparticles using green alga *Botryococcus braunii* and its antimicrobial activity. Bioinorg Chem Appl 9: 7879403.

Asghar MA, Zahir E, Shahid SM. 2018. Iron, copper and silver nanoparticles: green synthesis using green and black tea leaves extracts and evaluation of antibacterial, antifungal and aflatoxin B_1 adsorption activity. LW 90: 98-107.

Bonde S. 2011. A biogenic approach for green synthesis of silver nanoparticles using extract of *Foeniculum vulgare* and its activity against *Staphylococcus aureus* and *Escherichia coli*. Nusantara Biosci 3: 59-63. DOI: 10.13057/nusbiosci/n030201.

Brust M, Kiely CJ. 2002. Some recent advances in nanostructure preparation from gold and silver particles: A short topical review. Colloids Surf A: Physicochem Eng Asp 202 (2-3): 175-186. DOI: 10.1016/s0927-7757(01)01087-1.

Chakraborty N, Banerjee J, Chakraborty P, Banerjee A, Chanda C, Ray K. 2022. Green synthesis of copper/copper oxide nanoparticles and their applications: A review. 15: 187-215. DOI: 10.1080/17518253.2022.2025916.

Chandraker SK, La M, Ghosh MK, Tiwari V, Ghorai TK, Shukla R. 2020. Green synthesis of copper nanoparticles using leaf extract of *Ageratum houstonianum* Mill. and study of their photocatalyticand antibacterial activities. Nano Express 1: 010033. DOI: 10.1088/2632-959X/ab8e99.

Chung IM, Rahuman A, Marimuthu S. 2017. Green synthesis of copper nanoparticles using *Eclipta prostrata* leaves extract and their antioxidant and cytotoxic activities. Exp Ther Med 14: 18-24. DOI: 10.3892/etm.2017.4466.

Dadgostar N. 2008. Investigations on Colloidal Synthesis of Copper Nanoparticles in a Two-phase Liquid-liquid System. [Thesis]. University of Waterloo, Waterloo, Ontario. [Canada].

Delma MT, Rajan MJ. 2016. Green synthesis of copper and lead nanoparticles using *Zingiber officinale* stem extract. Intl J Sci Res Pub 6: 134-137.

Ebrahimi K, Shiravand H, Mahmoudvand H. 2017. Biosynthesis of copper nanoparticles using aqueous extract of *Capparis spinosa* fruit and investigation of its antibacterial activity. MARMARA Pharm J 21 (4): 866-871. DOI: 10.12991/mpj.2017.31.

Georgopoulos PG, Roy A, Yonone-Lioy MJ, Opiekun RE, Lioy PJ. 2001. Environmental copper: Its dynamics and human exposure issues. J Toxicol Environ Health B 4: 341-394. DOI: 10.1080/109374001753146207.

Ghaderian SM, Ravandi AAG. 2012. Accumulation of Copper and other heavy metals by plants growing on Sarcheshmeh Copper Mining Area, Iran. J Geochem Expl 2012: 25-32. DOI: 10.1016/J.GEXPLO.2012.06.022.

Gnanasangeetha D, Prathipa V. 2019. Zinc oxide nanoparticles for water remediation in agriculture. Intl J Mech Eng Technol 10 (1): 1547-1554.

Gnanasangeetha D, Sarala D. 2015. Green synthesis of benovolent ZnO narods using *Emblica officinalis*. Asian J Chem 27 (8): 3054-3056. DOI: 10.14233/ajchem.2015.18870.

Gnanasangeetha, D, Suresh M. 2020. A review on green synthesis of metal and metal oxide nanoparticles. Nat Environ Pollut Technol Intl Quart Sci J 19 (5): 1789-1800 DOI: 10.46488/NEPT.2020.v19i05.002.

Hailemariam G. 2011. Kinetic Study of Silver Ions Bioreduction for the Synthesis of Silver Nanoparticles and their Antibacterial Activity. [Thesis]. Haramaya University, Haramaya. [Ethiopoia]

Issaabadi Z, Nasrollahzadeh M, Sajadi MS. 2017. Green synthesis of the copper nanoparticles supported on bentonite and investigation of its catalytic activity. J Clean Prod 1: 3584-3591. DOI: 10.1016/j.jclepro.2016.10.109.

Jadoun S, Arif R, Jangid NK, Meena RK. 2021. Green synthesis of nanoparticles using plant extracts: A review. Environ Chem Lett 19: 355-374. DOI: 10.1007/s10311-020-01074-x.

Jaehoon L, Kim DK, Kang W. 2006. Preparation of ClI Nanoparticles from ClI Powder Dispersed in 2-Propanol by Laser Ablation. Bull Kor Chem Soc 27: 1869. DOI: 10.5012/bkcs.2006.27.11.1869.

Jeong J, Woo S, Kim D, Lim S, Kim JS, Shin H, Xia Y. 2008. Moon controlling the thickness of the surface oxide layer on Cu nanoparticles for the fabrication of conductive structures by ink-jet printing. Adv Func Mater 18 (5): 679-686. DOI: 10.1002/adfm.200700902.

Joseph AT, Prakash P, Narvi SS. 2016. Phytofabrication and characterization of copper nanoparticles using *Allium sativum* and its antibacterial activity. Intl J Sci Eng Technol 4: 463-472.

Kalainila P, Subha V, Ravindran RSE, Sahadevan R. 2014. Synthesis and characterization of silver nanoparticle from *Erythrina indic*. Asian J Pharm Clin Res 7 (2): 39-43.

Kaur P, Thakur R, Chaudhury A. 2016. Biogenesis of copper nanoparticles using peel extract of *Punica granatum* and their antimicrobial activity against opportunistic pathogens. Green Chem Lett Rev 9: 33-38. DOI: 10.1080/17518253.2016.1141238.

Khani R, Roostaei B, Bagherzade G, Moudi M. 2018. Green synthesis of copper nanoparticles by fruit extract of *Ziziphus spina-christi* (L.) Willd.: Application for adsorption of triphenylmethane dye and antibacterial assay. J Mol Liq 255: 541-549. DOI: 10.1016/j.molliq.2018.02.010.

Khodaie M, Ghasemi N. 2018. Green synthesis and characterization of copper nanoparticles using *Eryngium campestre* leaf extract. Bulgarian Chem Commun 50: 244-250.

Michael GEH. 2012. Biosynthesis of Copper Nanoparticles Using Some Plant Leaf Extracts, their Characterization and Antibacterial Activity. [Thesis]. Haramaya University. Haramaya. [Ethiopoia]

Moskovits M. Vlckova B. 2005. Adsorbate-induced silver nanoparticle aggregation kinetics. J Phys Chem B 109 (31): 14755-14758. DOI: 10.1021/jp051177o.

Murthy HC, Desalegn A, Kassa T, Abebe B, Assefa T. 2020. Synthesis of green copper nanoparticles using medicinal plant *Hagenia abyssinica* (Brace) JF. Gmel. leaf extract: Antimicrobial properties. J Nanomater 2020: 3924081.

Muthulakshmi L, Rajini N, Nellaiah H, Kathiresan T, Jawaid M, Rajulu AV. 2017. Preparation and properties of cellulose nanocomposite films with in situ generated copper nanoparticles using *Terminalia catappa* leaf extract. Intl J Biol Macromol 95: 1064-1071. DOI: 10.1016/j.ijbiomac.2016.09.114.

Nasrollahzadeh M, Mohammad SS. 2015. Green synthesis of copper nanoparticles using *Ginkgo biloba* L. leaf extract and their catalytic activity for the Huisgen [3 + 2] cycloaddition of azides and alkynes at room temperature. J Colloid Interf Sci 457: 141-147. DOI: 10.1016/j.jcis.2015.07.004.

Nasrollahzadeh M, Momeni SS, Sajadi SM. 2017. Green synthesis of copper nanoparticles using *Plantago asiatica* leaf extract and their application for the cyanation of aldehydes using K4Fe(CN)6. J Colloid Interf Sci 506: 471-477. DOI: 10.1016/j.jcis.2017.07.072.

Rajesh KM, Ajitha B, Reddy AK, Suneetha Y, Reddy PR. 2018. Assisted green synthesis of copper nanoparticles using *Syzygium aromaticum* bud extract: Physical, optical and antimicrobial properties. Optik 154: 593-600. DOI: 10.1016/j.ijleo.2017.10.074.

Rupareli JP, Chatterjee AK, Duttagupta SP, Mukherji S. 2008. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomaterialia 4: 707-771. DOI: 10.1016/j.actbio.2007.11.006.

Sable N, Gaikwad S, Bonde S, Gade A, Rai M. 2012. Phytofabrication of silver nanoparticles by using aquatic plant *Hydrilla verticillata*. Nusantara Biosci 4: 45-49. DOI: 10.13057/nusbiosci/n040201.

Saif S, Tahir A, Chen Y. 2016. Green synthesis of iron nanoparticles and their environmental applications and implications. Nanomaterials 6: 209. DOI: 10.3390/nano6110209.

Sarala P. 2015. Surficial geochemical exploration methods. In: Mayer WD, Lahtinen R, O'Brien H (Eds.). Mineral Deposits of Finland. Elsevier, Amsterdam. DOI: 10.1016/B978-0-12-410438-9.00027-3.

Sarkar J, Chakraborty N, Chatterjee A, Bhattacharjee A, Dasgupta D, Acharya K. 2020. Green synthesized copper oxide nanoparticles ameliorate defence and antioxidant enzymes in *Lens Culinaris*. Nanomaterials 2020: 10. DOI: 10.3390/nano10020312.

Shende S, Ingle AP, Gade A, Rai M. 2015. Green synthesis of copper nanoparticles by *Citrus medica* Linn. (Idilimbu) juice and its antimicrobial activity. World J Microbiol Biotechnol 31: 865-873. DOI: 10.1007/s11274-015-1840-3.

Thakur S, Sharma S, Rai R. 2018. Green synthesis of copper nanoparticles using *Asparagus adscendens* Roxb. root and leaf extract and their antimicrobial activities. Intl J Curr Microbiol Appl Sci 7 (4): 683-694. DOI: 10.20546/ijcmas.2018.704.077.

Theivasanthi T, Alagar M. 2011. Nano sized copper particles by electrolytic synthesis and characterizations. Intl J Phys Sci 6 (15): 3726-3735.

Valodkar M, Jadeja RN, Thounaojam MC, Devkar RV, Thakorea S. 2011. Biocompatible synthesis of peptide capped copper nanoparticles and their biological effect on tumor cells. Mater Chem Phys 128: 83-89. DOI: 10.1016/j.matchemphys.2011.02.039.

Yedurkar S, Maurya C, Mahanwar PA. 2017. Biological approach for the synthesis of copper oxide nanoparticles by *Ixora coccineas* leaf extract. J Mater Environ Sci 2017: 1173-1177.

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Incidence and knowledge of genotype and blood grouping among students as a guide to marriage counseling: A case study of FCE Katsina, Nigeria

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Abstract. *Mashi RL, Rafindadi MN, Lawal JY, Inusa YM. 2023. Incidence and knowledge of genotype and blood grouping among students as a guide to marriage counseling: A case study of FCE Katsina, Nigeria. Asian J Trop Biotechnol 20: 17-23.* Marriages have been formed over the years without the genotypes of the intended partners being known. As a result, many families have had children with sickle cell disease. This study examined the distributions of hemoglobin genotypes, ABO, and rhesus blood group patterns among Federal College of Education (FCE) Katsina, Nigeria, students as a reference to marital counseling. This was a cross-sectional study involving 200 students of FCE Katsina. The ABO blood group was determined using the tile method, while the hemoglobin genotype was determined using hemoglobin electrophoresis. Data analysis was done using a simple percentage. The results showed that out of the two hundred students that participated in this study, 146 (73%) of the subjects had HbAA, 51 (25.5%) had HbAS, 3 (1.5%) had HbAC, while there were none with HbSS genotype. The distribution of ABO blood groups of the subjects was; blood group O 114 (57%), blood group B 39 (19.5%), blood group A 39 (19.5%), blood group AB 5 (2.5%), A- 1 (0.5%) and O^- 2(1%). Furthermore, RhD positive was 197(98.5%), while RhD negative was 3 (1.5%). About 119 (59.5%) of the participants have not heard about the blood group, 147 (73.5%) do not know the Hb genotype and 171 (85.5%) are without Rhesus factor knowledge. This study has revealed knowledge and awareness gaps about Hb genotype, Blood group, Rhesus factor, sickle cell disease, and the distribution of ABO blood group amongst Federal College of Education Katsina students. Hence there is a need for massive marriage counseling and health education among students to reduce stillbirth due to Rhesus incompatibility and the burden of SCD, which has become a public health problem in our country.

Keywords: ABO, blood group, genotype, hemoglobin, Katsina, rhesus factor, sickle cell

INTRODUCTION

A blood genotype describes the genetic composition of a person's blood in its totality. No two people are the same. Persons may exhibit some resemblance, appear alike, act, and speak similarly, but no two people are completely identical. There are five different types of blood genotypes. They are as follows: AA, AS, AC, SS, and SC (Bougouma et al. 2012). While the first two pairs (AA and AS) are normal, AC is uncommon, and the last two (SS and SC) are irregular and abnormal, producing sickle cell disease (NIH 2002). Sickle cell disease occurs when an individual's blood cells are distorted and abnormally shaped, potentially limiting blood flow and causing pain and organ damage (Ilesanmi 2010). Before choosing a life mate, blood genotype is an important factor to consider. It is critical because the genotypes of the father and mother eventually cross to define the offspring's genotype (WHO 2006). Therefore, the appropriate knowledge of genotype compatibility can help someone make the optimal quality-of-life decision(s) about marriage and pregnancy. This help to avoid the catastrophic symptoms of sickle cell disease and, as a result, enhances the quality of life. In addition, the AA genotype has the highest compatibility ratio (Omuemu et al. 2013; Zounon et al. 2015).

Individuals with the AA genotype can pick a life partner from nearly all other genotype groups with a very low chance of having sickle-celled kids. According to Williams et al. (2005), while the AA genotype is the most compatible, it is also the most susceptible to malaria. The genotype is an individual's genetic composition, but the phenotype or blood group is the visible trait. The genotype is decided by the kind of hemoglobin found on red blood cells, while the phenotype is established by antigens found on red blood cells, leucocytes, and platelets, as well as antibodies found in plasma. When defined genetically, there are three fundamental sorts of people: those who inherited normal hemoglobin, those who inherited both normal and abnormal hemoglobin, and those who inherited the abnormal hemoglobin solely. As observed in sickle cell syndrome, the inheritance of defective hemoglobin in double dosage exposes one to various hereditary illnesses (Kohne 2011). The phenotypes are hereditary features that aid incompatible blood transfusion, compatible marital union, and treatment of pregnancies caused by incompatible marital unions (Kaine and Udeozo 1981;

Timothy 2000).

One's blood group is determined through genetics, which implies that it is handed on from parents to offspring. It refers to all antibodies (the body's natural defense), antigens (substances that induce the immune system to make antibodies against them), and other chemicals found on the surface of Red Blood Cells (Woldu et al. 2022). The ISBT (International Society of Blood Transfusion) now recognizes approximately 35 blood types; however, only four are widely used. These are A, B, AB, and O blood categories.

In 1941, Landsteiner and Weiner developed the Rhesus (Rh) blood classification (Firkin et al. 1989). The Rh blood type is a multi-antigen system with over 50 antigen expressions expressed by three pairs of tightly connected allelic genes on chromosome 1. However, the most important Rh antigens in medicine are D, C, E, c, and e. (Jahanpour et al. 2017). People with the Rh-D antigen are known as Rh-D positive, whereas those who do not have the antigen are said to be Rh-D negative. If an Rh-D negative individual receives Rh-D positive blood transfusion or an Rh-D negative mother carries an Rh-D positive fetus, the immune system recognizes the antigen as a foreign substance and synthesizes an antibody. That results in a hemolytic transfusion reaction and hemolytic disease in the fetus and newborn (Suresh et al. 2015).

Marriages have been formed over the years without the genotypes of the intended partners being known. As a result, many families have given birth to children. Moreover, the children have died due to neglect or ignorance of understanding their genotype before marriage. This was one of the primary issues for several couples in Nigeria, particularly in the north (Aderotoye-Oni et al. 2018). As a result, this study looked at the distributions of hemoglobin genotypes, ABO, and rhesus blood group patterns among FCE katsina students for marriage counseling.

MATERIALS AND METHODS

Study area

The study was conducted in the Federal College of Education (FCE) located in Katsina local government of Katsina State, Northern Nigeria (Figure 1). Katsina State is located between latitudes 11°08′N and 13°22′N and longitudes 6°52′E and 9°20′E. The state covers an area of 23,938 km^2 and lies in Northern Nigeria within the Sahel Savannah region. The state is bordered by Niger Republic to the North, Jigawa and Kano States to the East, Kaduna State to the South, and Zamfara State to the West. The state has 34 local government areas.

Sample size determination

The sample size is calculated using the Daniel formula (Daniel 1999).

$$n = \frac{Z^2 pq}{d^2}$$

Where: n = sample size, z = z statistics for a level of confidence of 95%, which is conventional, z value is 1.96; p = proportion of people with good knowledge of SCD; q = 1 - p; d = precision; z = 1.96; P = 17% (0.17); d = 0.05

$$n = \frac{(1.96)^2 \times 0.17(1-0.17)}{(0.05)^2}$$

$$\frac{3.84 \times 0.17 \times 0.83}{0.0025}$$

$$\frac{0.5}{0.0025}$$

$$n = 200$$

Two hundred respondents were recruited for the study.

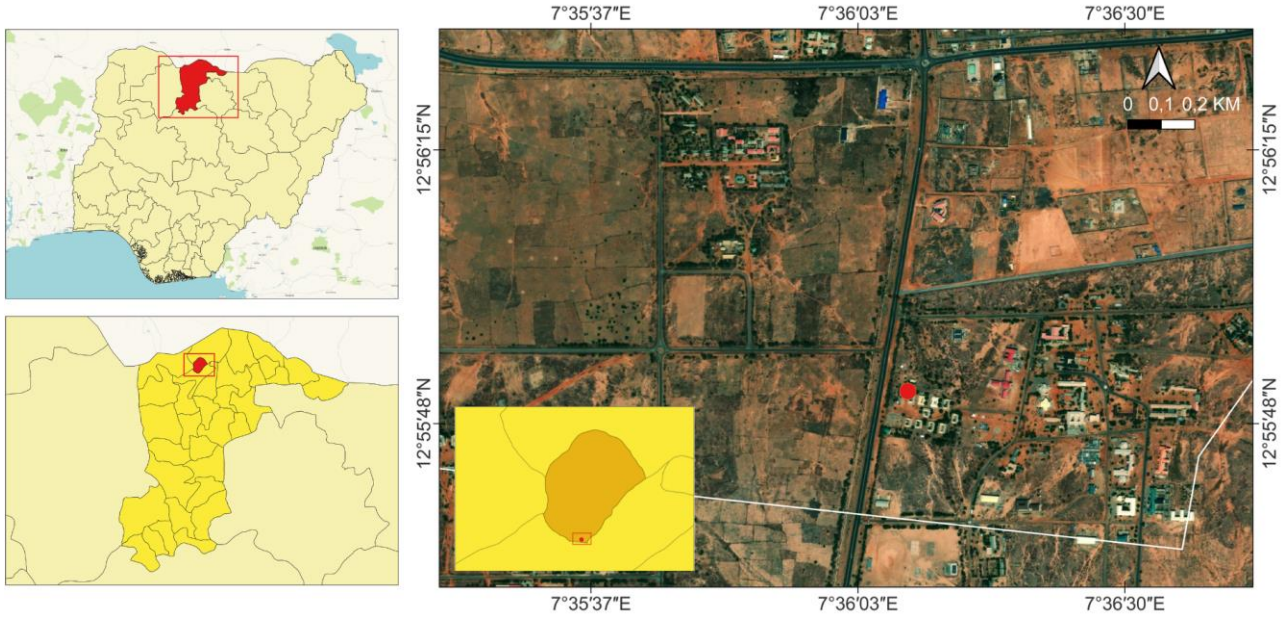

Figure 1. Federal College of Education in Katsina, Katsina State, Nigeria

Study population

A total of 200 study participants aged from 18 years up to 50 years were included. Furthermore, a pre-tested structured questionnaire was employed to collect sociodemographic data, and research participants were requested to provide informed permission. Respondents were randomly selected from third-year students who willingly submitted for the study. All the information was collected anonymously so that no respondent information could be revealed or identified for ethical reasons.

Sample collection

Exactly 2 mL of whole blood was collected aseptically from each subject using the standard vein puncture method. The blood sample was emptied into an EDTA (Ethylene Diamine Tetra-acetic Acid) container. It was properly mixed to avoid clotting and then used to determine hemoglobin, genotype, and blood grouping.

Determination of genotype

Hemoglobin electrophoresis was used to identify the genotype (Ochei and Kolhatkar 2008). Because Hbs are proteins, the migration rate of different hemoglobin (Hb) forms varies. Depending on the charge the Hb carries, they may be arranged to flow to either anode or cathode. The charge carried by hemoglobin is determined by the buffer employed (Hb). For example, when alkaline cellulose acetate is utilized in an alkaline buffer with a pH of 8.4, the Hb prefers to migrate to the anode, where there is a negative charge (Ochei and Kolhatkar 2008).

Each patient's venous blood hemolysate was deposited on the cellulose acetate membrane before being gently injected into the electrophoresis tank containing Tris - EDTA - Borate buffer at pH 8.6. The electrophoresis was then allowed to continue for 15 - 20 minutes at a voltage of 160 V. The findings were immediately read. As controls, haemolysates from known hemoglobin (AA, AS, AC) blood samples were used.

Determination of blood group

The tile and tube procedures were employed to determine the blood group (Karle Lanstiner 1901).

Tile method: On a clean tile, one drop of whole blood was deposited, and an equivalent amount of commercially produced standard antiserum (anti-A, anti-B, anti-D) was mixed in. After 2 minutes of incubation at room temperature, the observations were microscopically recorded using the naked eye.

Tube Method: The tube method was carried out for tile using cells, and the result was recorded for the tile grouping method. ABO and Rh-D blood group information was gathered utilizing a data-gathering methodology. To maintain the quality of the results, known positive and negative A-cell, B-cell, and Rh-D cells were employed.

Ethical consideration

Permission was obtained from the ethics committees of the Federal College of Education, Katsina State, Nigeria. Informed consent was obtained from the participants before sample collection. Refer to the appendix for the details of the questionnaire and consent form administered to the respondents in hard copy.

Data analysis

The data obtained were analyzed using percentages (%) and presented in tables and bar charts.

RESULTS AND DISCUSSION

Results showed that out of 200 participants, 130 (65%) were male, while 70 (35%) were female. The majority of the participants, 117 (58.5%), fall within the age bracket of 23-27 years, 68 (34%) aged 18-22 years, 10 (5%) aged 28-32 years, and 5 (2.5%) aged 33 years and above and larger proportion of the participants were single, 192 (96%), and only 8 (4%) were married as shown in Figure 2.

Table 1 shows the distribution of O^+, A^+, B^+, AB^+, A^- , and O^- blood groups among the participants as 114 (57%), 39 (19.5%), 39 (19.5), 5 (2.5%), 1 (0.5%) and 2 (1%) respectively, with Rhesus D positive (98.5%) and Rhesus' D' negative (1.5%). The distribution of the Hb genotype of HbAA 146 (73%), HbAS 51 (25.5%), and HbAC 2 (1%), respectively.

Table 2 shows the distribution of Hb Genotype and Blood group by gender of the participants as Hb genotype of HbAA male 93 (46.5) and Female 53 (26.5), HbAS Male 36 (18%) and Female 15 (7.5%), HbAC Male 1 (0.5%) and Female 1(0.5%) respectively and O^+, A^+, B^+, AB^+, A^- and O^- blood groups among the participants as male 98 (39%) and Female 36 (18%), Male 19 (9.5%) and Female 20 (10%), male 25 (12.5%) and female 4 (7%), 5 (2.5%), 1 (0.5%) and 2 (1%) all males respectively.

The participant's knowledge of the Hb genotype, blood group, Rhesus factor, and SCD, as shown in Table 3, revealed that the majority of the participants have not heard about the Hb genotype (25%), blood group (40.5%), Rhesus factor (14.5%) and sickle cell disease (26.5) respectively. Furthermore, the majority, 163 (81.5%), were Hausa, 12 (6%) were Fulani, and 25 (12.5%) others, as shown in Figure 3.

Table 1. Distribution of Hb genotype, blood group, and Rhesus factor among the participants

Variables	Categories	Frequency	Percentages (%)
Hb genotype	AA	146	73
	AS	51	25.5
	AC	3	1.5
	SS	0	0
ABO	A^+	39	19.5
	B^+	39	19.5
	AB^+	5	2.5
	O^+	114	57
	A^-	1	0.5
	O^-	2	1
Rhesus	Positive	197	98.5
	Negative	3	1.5

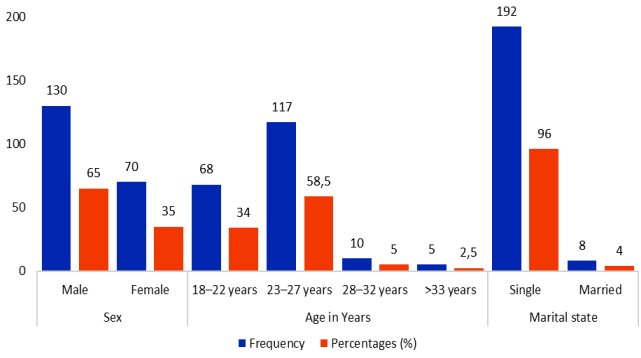

Figure 2. Distribution of gender, marital status, and age among the participants

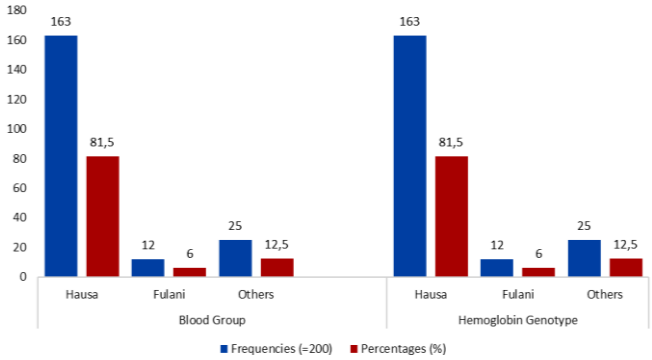

Figure 3. Distribution of blood group and hemoglobin genotype by ethnic group among the participants

Table 2. Distribution of Hb genotype, blood group, and Rhesus factor by gender of the participants

Variables		Males		Females		Total
		Freq.	Percent.	Freq.	Percent.	%
ABO	A^+	19	9.5	20	10	19.5
	B^+	25	12.5	14	7	19.5
	AB^+	5	2.5	0	0	2.5
	O^+	78	39	36	18	57
	A^-	1	0.5	0	0	0.5
	O^-	2	1	0	0	1
	Total	130	65	70	35	100
Hb genotype	AA	93	46.5	53	26.5	73
	AS	36	18	15	7.5	25.5
	AC	1	0.5	2	1	1.5
	SS	0	0	0	0	0
	Total	130	65	70	35	100

Table 3. Participants' knowledge of Hb genotype, blood group, Rhesus factor, and SCD

Variables	Labels	Frequencies (n=200)	Percentage (%)
Hemoglobin genotype	Yes	50	25
	No	150	75
Blood group	Yes	81	40.5
	No	119	59.5
Sickle cell disease	Yes	53	26.5
	No	147	73.5
Rhesus factor	Yes	29	14.5
	No	171	85.5

Discussion

The findings of this study revealed that out of the 200 participants, 130 (65%) were male, while 70 (35%) were female. This might not necessarily mean more male students are in the college where this study was conducted. Still, the male students were more interested in the study than the female since the individuals in this study were allowed to participate willingly. This low interest in the participation of females may be ascribed to the aspects of beliefs, intimidation of girls by boys, gender stereotyping in science, and scarcity of female models in science and technology. The majority of the participants, 117 (58.5%), fall within the age bracket of 22-27 years, 68 (34%) aged 18-22 years, 10 (5%) aged 28-32 years, and 5 (2.5%) aged 33 years and above, this is because most of the third year students were within this age brackets in the studied area. The majority, 163 (81.5%), were Hausa, 12 (6%) were Fulani, and 25 (12.5%) others, because the Hausa Tribe dominates the study area. A larger proportion of the participants were single, 192 (96%), and only 8 (4%) were married because most undergraduate students were single in the study area.

According to Ethiopian research on ABO distribution, the "O" blood type was the most prevalent, followed by the "A" and "B" blood groups (Atire 2015; Tesfaye et al. 2015; Tiruneh et al. 2020; Woldu et al. 2022). The proportion of people with the blood group "O" ranged from 21.67% to 47.04% (Abegaz 2021; Woldu et al. 2022). The "AB" blood group, on the other hand, was the least common blood group type, with a prevalence ranging from 2.87% to 24.7% (Abegaz 2021). The most frequent blood group was Rh-D positive, accounting for 92.77% to 60.13% of the population (Abegaz 2021). In the present study, the distribution of O^+, A^+, B^+, AB^+, A^- , and O^- blood groups among the students of Federal College of Education, Katsina, was 114 (57%) male, 98(39%), and Female 36 (18%), 39 (19.5%) Male 19 (9.5%) and Female 20 (10%), 39 (19.5) male 25 (12.5%) and female 14 (7%), 5 (2.5%), 1 (0.5%) and 2 (1%) all males respectively. The research indicated that the "O" blood type was the majority blood group, followed by A and B, AB, O^- , and the least prevalent blood group was A^-. This study is comparable to a study completed in Nigeria (Odokuma et al. 2007; Enosolease and Bazauye 2008; Erhabor et al. 2013), in Ethiopia (Zerihun and Bekele 2016; Golassa et al. 2017; Legese et al. 2021), in Bangladesh (Talukder and Das 2007) and India (Sharma et al. 2013). According to these studies, the "O" blood group was the most prevalent, while the "AB" blood group was the least common.

The Rh D positive blood group was most common in this study accounting for 98.5%; this is similar to the work of Erhaboe et al. (2013) and Legese et al. (2021). Equally, the frequency of Rhesus-positive antigen in this study, 98.5%, is also in the same range as 96.7% recorded for the Ibos by Ukaejiofor et al. (1996) and similar to 97.7% found

in Port Harcourt by Jeremiah (2005). It is a striking result in this study that 1.5% of Rhesus negatives were all males. This percentage of Rhesus negative observed in our study (1.5%) is significantly lower than the prevalence rate of >14% Rh (D) negative phenotype observed in studies among Caucasians (Cerny et al. 1992). Several obstetric advantages are associated with the low prevalence of D-negative among the studied population in Katsina. Therefore, the risk of Rh (D) alloimmunization will be of a much smaller magnitude than it is in most Western countries, where a significant proportion of the population lacks the major Rh (D) antigen (Erhabor et al. 2013).

However, this study is in contrast with a study conducted in Ethiopia which showed blood group A in the dominant (Abegaz 2021) and in India (Barot et al. 2020) and Pakistan (Pramanik and Pramanik 2000; Khattak et al. 2008) which showed blood group B is dominant than O group. The disparity between our findings and those of other research might be due to variations in sample size, sampling procedure, and genetic diversity between ethnic groups within other countries' populations.

The frequencies of the HbAA 146 (73%) male 93 (46.5) and Female 53 (26.5), HbAS 51(25.5%) Male 36 (18%) and Female 15 (7.5%), HbAC 2 (1%) Male 1 (0.5%) and Female 1(0.5%), respectively. Our finding is similar to the work of Kaine and Udeozo (1981), who got HbAS of 25% and HbAC of 0.1% in Igbo land, southeastern Nigeria. The result also agrees with Afolayan and Jolayemi's (2011) work, which found 25% of sickle cell trait prevalence in Nigeria. Although the prevalence of HbAA was the highest, the presence of the HbAS and HbAC among the studied population may suggest the possibility of many other residents in the capital city of Katsina State carrying abnormal forms of hemoglobin genotype. Therefore, that could call for more efforts in genetic counseling in this area. The prevalence of HbAS and HbAC above other abnormal hemoglobin genotypes HbSS, HbSC, and HbCC in this study could be justified by the presence of one normal allele coding for a normal polypeptide globin chain which confers on the hemoglobin better affinity for oxygen. Hence, individual carriers of these abnormal hemoglobin genotypes still live a normal life, unlike in the case of HbSS and HbCC (Akinboro et al. 2016).

This study shows poor knowledge of Hb genotype (25%), blood group (40.5%), Rhesus factor (14.5%), and sickle cell disease (26.5%), respectively, by the participants. This is similar to research by Adewuyi (2000) in Ilorin, Nigeria, who reported poor knowledge among fresh graduate students. Similarly, Bazuaye and Olayemi (2009) also reported in their study that more than half of the participants in Benin City did not know their genotype and only a few respondents had good knowledge about sickle cell disease. The participants' lack of understanding of the sickle cell genotype and the Rhesus antigen was unexpected, given their level of education. This finding demonstrates how little awareness numerous people in Katsina may have regarding sickle cell illness and neonatal hemolytic anemia. Nevertheless, due to cultural views in the investigated region, the conclusion might be ascribed to a lack of awareness or an unwillingness to embrace health-enlightening programs.

In conclusion, this study has revealed knowledge and awareness gaps about the Hb genotype, Blood group, Rhesus factor, and sickle cell disease, and the distribution of the ABO blood group was O>A>B>AB, while the genotype was HbAA>HbAS>HbAC, respectively amongst the students of Federal College of Education Katsina. Hence there is a need for massive marriage counseling and health education among students to reduce stillbirth due to Rhesus incompatibility and the burden of SCD, which has become a public health problem in our country.

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REFERENCES

Abegaz SB. 2021. ABO and Rh (D) blood group distribution among blood donors: A study of natural and computational science faculty graduating class students at Woldia University, Ethiopia. Intl J Genet Mol Biol 13 (1): 21-30. DOI: 10.5897/IJGMB2020.0203.

Aderotoye-Oni S, Diaku-Akinwumi IN, Adeniran A, Falase B. 2018. Unprepared and misinformed parents of children with sickle cell disease: Time to rethink awareness campaigns. Cureus 10 (12): e3806. DOI: 10.7759/cureus.3806.

Adewuyi JO. 2000 Knowledge of and attitude to sickle cell disease and sickle carrier screening among new graduates of Nigerian Tertiary Educational Institutions. Niger Postgrad Med J 7 (3): 120-123.

Afolayan JA, Jolayemi FT. 2011. Parental attitude to children with sickle cell disease in selected health facilities in Irepodun Local Government, Kwara State Nigeria. Stud Ethno-Med 5 (1): 33-40. DOI: 10.1080/09735070.2011.11886389.

Akinboro A, Komofale EK, Azeez MA. 2016. A retrospective study on fourteen year hemoglobin genotype variants recorded at five government hospitals in Akure, Ondo State, Southwestern Nigeria. Egypt J Med Hum Genet 17 (4): 377-381. DOI: 10.1016/j.ejmhg.2016.02.005.

Atire FA. 2015. Distribution of ABO and Rh blood groups among students of some ethnic groups at Dilla University, Ethiopia. Intl J Genet Genomics 3 (1): 8-19. DOI: 10.11648/j.ijgg.20150301.12.

Barot T, Patel D, Shah R. 2020. Distribution of ABO and Rhesus (Rh) blood groups among voluntary blood donors in Central Gujarat, India. Intl J Contemp Med Res 7 (7): G1-G3. DOI: 10.21276/ijcmr.2020.7.7.24.

Bougouma EC, Tiono AB, Ouédraogo A, Soulama I, Diarra A, Yaro JB, Ouédraogo E, Sanon S, Konaté AT, Nébié I, Watson NL, Sanza M, Dube TJT, Sirima SB. 2012. Hemoglobin variants and Plasmodium falciparum malaria in children under five years of age living in a high and seasonal malaria transmission area of Burkina Faso. Malar J 4 (11): 154. DOI: 10.1186/1475-2875-11-154.

Cerny T, Fey MF, Oppliger R, Castiglione M, Nachbur B, Gertsch M, Gasser A, Joss RA, Thatcher N, Lind M et al. 1992. Prevalence of the Rhesus-negative phenotype in Caucasian patients with Small-Cell Lung Cancer (SCLC). Intl J Cancer 52 (3): 504-506. DOI: 10.1002/ijc.2910520328.

Daniel WW. 1999. Biostatistics: A Foundation for Analysis in the Health Sciences. 7th ed. John Wiley and Sons, New York.

Enosolease ME, Bazuaye GN. 2008. Distribution of ABO and Rh-D blood groups in the Benin area of Niger-Delta: Implication for regional blood transfusion. Asian J Transfus Sci 2 (1): 3-5. DOI: 10.4103/0973-6247.39502.

Erhabor O, Isaac IZ, Saidu A, Ahmed HM, Abdulrahaman Y, Festus A, Ikhuenbor DB, Iwueke IP, Adias TC. 2013. The distribution of ABO and Rhesus blood groups among residents of Gusau, Zamfara State, North Western Nigeria. Res Rev J Med Health Sci 2 (4): 58-63.

Firkin F, Chesterman C, Penington D, Rush B. 1989. de Gruchy's Clinical Hematology in medical practice. In: Blood Groups; Blood Transfusion; Acquired Immune Deficiency Syndrome. 5th ed. Oxford University Press, NewDelhi.

Golassa L, Tsegaye A, Erko B, Mamo H. 2017. High Rhesus (Rh(D)) negative frequency and ethnic-group based ABO blood group distribution in Ethiopia. BMC Res Notes 10 (1): 330. DOI: 10.1186/s13104-017-2644-3.

Ilesanmi OO. 2010. Pathological basis of symptoms and crises in sickle cell disorder: Implications for counseling and psychotherapy. Hematol Rep 2 (1): e2. DOI: 10.4081/hr.2010.e2.

Jahanpour O, Pyuza JJ, Ntiyakunze EO, Mremi A, Shao ER. 2017. ABO and Rhesus blood group distribution and frequency among blood donors at Kilimanjaro Christian Medical Center, Moshi, Tanzania. BMC Res Notes 10 (1): 738. DOI: 10.1186/s13104-017-3037-3.

Jeremiah ZA. 2005. Abnormal hemoglobin variants, ABO and Rh blood groups among student of African descent in Port Harcourt, Nigeria. Afr Health Sci 6 (3): 177-181. DOI: 10.5555/afhs.2006.6.3.177.

Kaine WN, Udeozo IO. 1981. Incidence of sickle cell trait and Anemia in Igbo Preschool Children. Niger J Paediatr 8 (4): 87-89.

Khattak ID, Khan TM, Khan P, Shah SMA, Khattak ST, Ali A. 2008. Frequency of ABO and Rhesus blood groups in District Swat, Pakistan. J Ayub Med Coll Abbottabad 20 (4): 127-129.

Kohne E. 2011. Hemoglobinopathies: Clinical manifestations, diagnosis, and treatment. Dtsch Arztebl Intl 108 (31-32): 532-540. DOI: 10.3238/arztebl.2011.0532.

Legese B, Shiferaw M, Tamir W, Tiruneh T. 2021. Distribution of ABO and Rhesus blood group phenotypes among blood donors at Bahir Dar Blood bank, Amhara, Northwest Ethiopia: A retrospective cross-sectional study. J Blood Med 12: 849-854. DOI: 10.2147/JBM.S329360.

National Institute for Health (NIH). 2002. Genetic disease profile; sickle cell Anemia NIH Publications No. 96 - 4057. Nov2002. http://www.ornl.gov/techresources/humanGenone/posters/chromosome/SCA.html.

Ochei J, Kolhatkar, A. 2008. Miscellaneous Investigation in Hematology. Medical Laboratory Science, Theory and Practical Data. Mc Graw-Hill, New York.

Odokuma EI, Okolo AC, Aloamaka PC. 2007. Distribution of ABO and rhesus blood groups in Abraka, Delta state. Niger J Physiol Sci 22 (1-2): 89-91.

Omuemu VO, Obarisiagbon OE, Ogboghodo EO. 2013. Awareness and acceptability of premarital screening of sickle cell disease among undergraduate students of the University of Benin, Benin City, Edo State. J Med Biomed Res 12 (1): 91-104.

Pramanik T, Pramanik S. 2000. Distribution of ABO and Rh blood groups in Nepalese medical students: A report. East Mediterr Health J 6 (1): 156-158. DOI: 10.26719/2000.6.1.156.

Sharma DC, Rai S, Iyenger S, Jain B, Sao S. 2013. Prevalence and distribution of ABO and Rh-D antigens along with its subgroups & rare types in Greater Gwalior Region. Open J Blood Dis 3: 69-73. DOI: 10.4236/ojbd.2013.32015.

Suresh B, Babu SKV, Mouli PC, Arun R, Jothibai DS. 2015. Distribution of ABO and Rhesus (D) blood group antigens among blood donors at a tertiary care teaching hospital blood bank in South India. J Clin Sci Res 4: 129-135. DOI: 10.15380/2277-5706.JCSR.14.064.

Talukder SI, Das RK. 2010. Distribution of ABO and Rh blood groups among blood donors of Dinajpur District of Bangladesh. Dinajpur Med Col J 3 (2): 55-58.

Tesfaye K, Petros Y, Andargie M. 2015. Frequency distribution of ABO and Rh (D) blood group alleles in Silte Zone, Ethiopia. Egypt J Med Hum Genet 16 (1): 71-76. DOI: 10.1016/j.ejmhg.2014.09.002.

Timothy AW. 2000. Gastrointestinal manifestations of sickle cell disease. http://www.dcmsonline.org/jaxmedicine/2000journals/june2000/gastro.html.

Tiruneh A, Yetneberk T, Gelaw M, Eshetie D. 2020. Frequency of ABO and Rh blood group distribution at Debre Tabor blood bank, Amhara Region, North-Central Ethiopia. A six-year retrospective survey. J Blood Med 11: 357. DOI: 10.2147/JBM.S266624.

WHO. 2006. Sickle cell anemia. Fifty-Ninth World Health Assembly. 2006; A59/ 9(April):1-5. Available at: http://apps.who.int/iris/bitstream/handle/10665/20890/A59_9-en.pdf?sequence=1&isAllowed=y last accessed Nov, 2018.

Williams TN, Mwangi TW, Roberts DJ, Alexander ND, Weatherall DJ, Wambua S, Kortok M, Snow RW, Marsh K. 2005. An immune basis for malaria protection by the sickle cell trait. PLoS Med 2 (5): e128. DOI: 10.1371/journal.pmed.0020128

Woldu B, Melku M, Shiferaw E, Biadgo B, Abebe M, Gelaw Y. 2022. Phenotype, Allele and genotype frequency of ABO and Rhesus D blood groups of blood donors at the North Gondar district blood bank, Northwest Ethiopia. J Blood Med 13: 11-19. DOI: 10.2147/JBM.S346904.

Zerihun T, Bekele S. 2016. Pattern of ABO and Rhesus blood groups distribution of five years survey in Jimma Town blood bank, South West Ethiopia. J Health Educ Res Dev 4: 1-4. DOI: 10.4172/2380-5439.1000177.

Zounon O, Sorum PC, Mullet E. 2015. How people in Benin assess a couple's risk of having a baby with sickle cell disease. J Community Genet 6 (1): 77-82. DOI: 10.1007/s12687-014-0205-1.

Appendix

RESEARCH QUESTIONNAIRE

Sociodemographic characteristics
Age/date of birth ……..….
Gender: Male [ ], Female [ ]
Marriage [ ], Single [ ]
Ethnic group: Hausa [ ], Fulani [ ], Yoruba [ ], Igbo [ ], others …….…..

Knowledge of blood group and genotype
1. Did you know anything about Hb genotype? Yes [ ], No [ ]
2. Did you know anything about Blood group? Yes [ ], No [ ]
3, Did you know anything about Sickle cell anemia? Yes [ ], No [ ]
Did you know anything about Rhesus factor? Yes [ ], No [ ]
Did you know anything about still birth? Yes [ ], No [ ]

Consent
Name ……
Age ……
Sex ……
Address……
Phone number……
E mail……
Occupation……

I, the undersigned hereby willingly wish to participate in the above study, and consent to collect my blood sample to assay for Blood group and genotype, having understood the questionnaire and the professional counseling by the researcher. I understand that I will be informed of the outcome of the findings on my sample.

Signature……
Date……
Time……

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Enterobacter tabaci and *Bacillus cereus* as biocontrol agents against pathogenic *Ralstonia solanacearum* of eggplant

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Abstract. *Malek AA, Ali NS, Kadir J, Vadamalai G, Saud HM. 2023.* Enterobacter tabaci *and* Bacillus cereus *as biocontrol agents against pathogenic* Ralstonia solanacearum *of eggplant. Asian J Trop Biotechnol 20: 24-30.* Over the world, *Ralstonia solanacearum* caused bacterial wilt disease is responsible for more than 90% of agricultural losses. Therefore, finding a potential biological control agent is extremely important, especially against *R. solanacearum.* The present study aimed to isolate, evaluate and identify potential endophytic bacteria to restrain *R. solanacearum* via in vitro antagonistic assay. A total of 116 endophytic bacteria were isolated, and 55 exhibited abilities to restrain *R. solanacearum* in vitro. However, only two isolates, UPMBC1 and UPMBE2, significantly inhibited R. solanacearum growth by 14.6 mm and 12.6 mm, respectively. Based on the cultural and morphological characteristics, UPMBC1 and UPMBE2 isolates exhibited similar characteristics as the other members of Enterobacteriaceae and Bacillaceae, respectively. Molecular results revealed that UPMBC1 was identified as *Enterobacter tabaci* and UPMBE2 as *Bacillus cereus*. Numerous efficient biological control agents were identified in the present investigation, with *E. tabaci* showing the greatest potential against *R. solanacearum.*

Keywords: Antagonistic assay, *Bacillus cereus*, bacterial wilt disease, *Enterobacter tabaci*, potent endophytic bacteria, *Ralstonia solanacearum*

INTRODUCTION

The eggplant originated from Indo-Burma, based on a Sanskrit document dated as early as 300 BCE (Daunay and Janick 2007). Later, it was brought to China, Japan, East Africa, and the Mediterranean Basin before spreading across the globe as time passed. Eggplant production in Malaysia showed a progressive trend, increasing from 39,103 metric tons (mt) in 2019 to 42,809 mt in 2021 (*Jabatan Pertanian* 2021). The statistical data also reported that Malaysia had achieved over 100% self-sufficiency in eggplant production.

Ralstonia solanacearum (Smith) can infect more than 200 different crop species worldwide that cover 50 families (Achari and Ramesh 2014; Hemelda et al. 2019). Its infection occurs in tropical, sub-tropical, and temperate climates worldwide, with profound severity noted in tropical climate countries (Mondal et al. 2014). The pathogen is capable of destroying crops and causing significant yield losses of up to 90% (Artal et al. 2012). The prominent effects of Bacterial Wilt (BW) disease can be observed in tomatoes, bananas, eggplant, and potatoes, with severity ranging from 80% to 100%. In Ethiopia, 55% of wilt incidence was recorded on tomatoes, which greatly reduced its production and affected the production of other solanaceous crops (Yadessa et al. 2010). In India, BW disease was a major constraint on tomatoes and eggplant in major-growing areas (Mondal et al. 2014). In southwestern Nigeria, 80% of the tomato yield was highly reduced due to BW infection (Popoola et al. 2014). Similar incidents were reported in banana cultivation in Malaysia, affecting 90% of the yields in 2014 (Zulperi and Sijam 2014). The first infection with BW disease was reported in Guyana in 1840 by Thurston and Galindo (1989). Later, Burrill discovered the causal agent when it infected the potato in Japan in 1890 (Tahat and Sijam 2010). Many ongoing studies have focused on finding the most effective control of *R. solanacearum* concerning the environment and human safety, as a pathogen is not easy to eradicate. The non-sporulate bacterium can survive for years in dry soil or the absence of the host (Stander et al. 2013). Infection would occur even if the pathogen population were low (Nair 2013). Moreover, *R. solanacearum* can persist for months or years in the host even after the host has died, providing inoculum for the next infection (Scherf et al. 2010). The variability of the strain and widely distributed hosts even make it difficult to resist the BW infection (Blomme et al. 2017).

Since there is no promising control for BW disease, farmers rely on the resistant cultivar to resist the infection (Artal et al. 2012). However, the resistant cultivar is also restricted to certain *R. solanacearum* strains. Alternatively, researchers switched to the biological control approach to control BW and found it effective in controlling this soil-borne disease (Lwin and Ranamukhaarachchi 2006). Endophytic Bacteria (EB) were known for their symbiotic relationship with the host plant and were widely used to suppress *R. solanacearum*. Generally, EB colonizes

internal plant tissues and exists in the root and xylem of plants. Extensive colonization by EB acts as a barrier to prevent the wilt pathogen from establishing in the vascular tissues (Bell et al. 1995). Some genera proved effective in suppressing the growth of *R. solanacearum*, such as *Streptomyces* sp. *Bacillus* sp., *Pseudomonas* sp., and *Enterobacter* sp. (Liu et al. 2009; Xue et al. 2009). EB's benefit the host plant by contributing to plant growth by fixing the nitrogen, solubilizing phosphate, and producing a siderophore for the iron uptake (Egamberdieva et al. 2017). In addition, EB acted as a root colonizer, capable of expelling the wilt pathogen during nutrient source competition (Toyota et al. 2000). EB's secretion of volatile compounds and metabolites could help the plant defense system (Tahir et al. 2017). In a nutshell, EB has the potential to act as a phytostimulant, biological control agent, biofertilizer, and soil remediation agent. However, it was hard to find novel and potent EB against *R. solanacearum* as this pathogen was genetically diverse, had a broad geographical distribution, and had long persistence in soil. Therefore, the objectives of this study were (i) to isolate and evaluate the virulence of potential EB against *R. solanacearum* and (ii) to characterize and identify the potent EBs against *R. solanacearum* via cultural, morphological, and molecular techniques.

MATERIALS AND METHODS

Isolation of pathogen and pathogenicity test

R. solanacearum was isolated from wilted eggplant on a small private farm in Kuala Selangor, Malaysia. The infected stem was recognized by the wilting symptom and thick slime running out when placed in water. Wilted stems were surface sterilized by dipping them in 20% sodium hypochlorite (NaOCl) for 30 seconds and then washing them three times with sterile distilled water. Next, 1 cm of each end of the wilted stem was discarded, while the remaining length was cross-sectioned and dipped in sterile distilled water to collect the bacterial slime (ooze). One loop of the infected distilled water was streaked on Kelman Tetrazolium Chloride (TZC) agar to confirm the pathogen and incubated for 48 hours at 25°C. The most virulent strain was chosen for the pathogenicity test. *R. solanacearum* was inoculated in Casein-Protease-Glucose (CPG) broth and shaken for 48 hours at 25°C at 140 rpm. The concentration was determined via spectrophotometer at 600 nm absorbance using Multiscan™ GO equipment (Thermo Scientific, Germany) and adjusted to 10^{10} CFU/mL. Ten replicates of 30-day-old commercial eggplant seedlings, Antaboga hybrid (F1), were soil-drenched with 30 mL of *R. solanacearum* inoculated CPG broth. The soil of the control pots was drenched with 30 mL of sterile distilled water. An adequate amount of water was applied to the seedlings twice daily. The seedlings were monitored for wilting symptoms for three weeks, and Disease Severity (DS) was calculated according to the formula of Winstead and Kelman (1952).

$$\text{Disease severity (DS) (\%)} = [\sum (ni \times v_i) \div (V \times N)] \times 100$$

Where:

ni : the number of plants with the respective disease rating v_i : disease rating

V : highest disease rating

N : number of plants observed.

The disease rating was measured on the following scale: 1: no symptoms; 2: one leaf wilted; 3: two-three leaves wilted; 4: more than 4 leaves wilted; and 5: the whole plant wilted.

Isolation of endophytic bacteria

Therefore, the healthy stems of eggplant, chili, and tomato (8-12 weeks old) were collected from seven states of Peninsular Malaysia: Johor, Selangor, Penang, Kedah, Perak, Pahang, and Negeri Sembilan. 11 cm of the main stem for each sample was excised, and the bark was discarded. The later, excised stem was dipped in 20% NaOCl for one minute and washed using sterile distilled water thrice. The washed water was streaked onto the Nutrient Agar (NA) as a control to ensure sterilization. One cm of each end of the stem was discarded, and the remaining stem was cut into 3 cm lengths horizontally to expose the interior part using a sterile blade. The pieces were transferred into a 200 mL 1×PBS (Phosphate Buffered Saline) buffer and shaken at 140 rpm for 48 hours at 25°C. After 48 hours, one loopful of EB-containing buffer was streaked on NA and incubated for four days at 25°C. After four days, different colonies of EB were selected based on their shape, color, colony, and texture. Subsequently, EB was purified onto fresh NA until a single colony was obtained. In addition, five replications were performed for each different crop.

In vitro antagonistic assessment

Purified EB (individually) and *R. solanacearum* were cultured in 5 mL of Nutrient Broth (NB) and CPG broth, respectively, and shaken at 140 rpm for 48 hours at 25°C. Next, 250 mL of NA was prepared, and 150 µL of *R. solanacearum* broth was added to every 100 mL of NA, swirled, and poured onto a Petri plate. Three wells were made for each plate using a sterile 1 cm cork borer (in diameter). Next, 25 µL of EB suspension was pipetted into each well, and the plates were sealed. In the control plates, distilled water was used. All plates were incubated for four days at 25°C, and the growth radius of EB (inhibition radius) was measured from the edge of the well in mm (direction: top, below, left, and right). Ten replications were performed for each EB. Isolates with the highest radius inhibition (>10 mm) were selected for further experiments.

Cultural and morphological characterization

The colony was characterized by evaluating the diameter, margin, pigmentation, opacity, texture, and form. In addition, Gram-staining was performed to observe the types of cell walls and cell shapes described by Tankeshwar (2015). The Gram-staining slide was observed on a light microscope Olympus CX31 RBSF (Olympus Corporation, Japan) under 40X, 100X, 400X, and 1000X magnification (using oil immersion). The images were

magnified using the Dino-Lite USB microscope camera AM 4515ZTL (AnMo Electronics Corporation, Taiwan) and captured using the Dino Capture 2.0 software (AnMo Electronics Corporation, Taiwan).

Molecular identification

Molecular identification was performed using the Polymerase Chain Reaction (PCR) method (Walch et al. 2016). Only EB with more than 10 mm of mean inhibition zone measurement in in vitro inhibition bioassay was subjected to molecular identification. Next, 50 µL of master mix for each EB was prepared. The master mix contained 1.25 µL of Taq polymerase, 1.0 µL of dNTPs, 2.5 µL each of 1429R (5' GGTTACCTTGTTACGACTT 3'), and 27F primers (5' AGAGTTTGATCCTGGCTCAG 3'), 5.0 µL of PCR buffer and 37.75 µL of sterile distilled water. A sterile 10 µL tip was used to dip slightly on the EB colony and mixed with the 50 µL master mix mixture. As for the control, no EB was added. Polymerase Chain Reaction tubes (0.5 mL) containing the prepared master mix were transferred into the PCR machine (c1000 Bio-Rad Thermal Cycler, United States) for amplification.

Next, 1% agarose gel was prepared using 1 × Tris-acetate-EDTA (TAE) buffer, microwaved, and poured into a casting tray. The gel was left to solidify for 20 minutes and subsequently transferred into an electrophoresis gel documentation system (Thermo Fisher Scientific, United States) containing 1 × TAE buffer (Appendix A). Next, 1 µL of 100 bp Thermo Fisher Scientific ladder was mixed with 1 µL loading dye and pipetted into the first well as a marker. Next, the amplified PCR products were pipetted into the wells of solidified agarose gel. Finally, 1 µL of PCR product was mixed with 1 µL of loading dye (Thermo Fisher Scientific) to give color. Electrophoresis was run for 45 minutes at 85 volts until the dye reached approximately 80% of the gel. The gel obtained from the electrophoresis was placed in the Bio-rad Gel Doc XR Imaging System (Bio-rad, United States) and viewed under Ultraviolet (UV) light for the band. Subsequently, the PCR products of each EB were sent to NHK Bioscience Solution Sdn. Bhd. for sequencing.

Analysis of Variance (ANOVA)

Statistical analysis was done using Tukey's Honest Significant Difference (HSD) with probability ($P<0.05$) and performed via the Statistical Analysis System (SAS) software version 9.4 (SAS Institute, United States).

RESULTS AND DISCUSSION

Isolation of pathogen and pathogenicity test

The isolation result showed that three different *R. solanacearum* strains were isolated based on cultural appearances. Among all three strains, only one strain produced a fluidal pinkish colony with a whitish margin, indicating a virulent strain (Figure 1A). The other isolated strains produced a deep red colony with a non-fluidal and non-whitish margin, indicating a non-virulent strain. In the pathogenicity test, eggplant showed low wilting in the first and second weeks with 20% Disease Severity (DS). However, in the third week, DS was accelerated to 80%. The symptoms were wilted green leaves remained attached to the stem. The comparison between infected and control eggplant seedlings in the pathogenicity test is shown in Figure 1B.

In vitro antagonistic assessment

Furthermore, a total of 116 isolates were obtained from healthy chili, eggplant, and tomato plants. The highest (39) isolates of EB were obtained from chili, while eggplant and tomato yielded the lowest EB with 11 isolates. Out of 116 isolates, 55 showed inhibition against *R. solanacearum* growth in in vitro antagonistic assessment. However, only 27 isolates were subjected to ANOVA analysis based on their consistency in inhibiting the growth of *R. solanacearum*. Based on the analysis, the highest (14.64 mm) mean radius of inhibition was observed by UPMBC1, followed by UPMBE2, with a mean radius of 12.55 mm. The UPMBC1 was isolated from chili in Selangor, while UPMBE2 was isolated from eggplant in Selangor. Among the 27 isolates, the highest, i.e. (7) isolates of potential EB, was recorded from Selangor. Nevertheless, chili and eggplant had the highest number of eight isolates. The growth inhibition of EB against *R. solanacearum* via in vitro antagonistic assessment is shown in Figure 2, and the inhibition radius is tabulated in Table 1.

Cultural and morphological characterizations

Enterobacter tabaci was cocci-shaped, and its cell wall was stained with pink safranin, which indicates a Gram-negative bacterium. On the Petri plate, the colony exhibited a round margin, smooth and translucent appearance, and yellowish coloration with a butyrous texture. The colony size was 2 mm in diameter; the *Bacillus cereus* was rod-shaped, and the cell wall was stained with purple-violet crystal color, indicating a Gram-positive bacterium. On the plate, *B. cereus* had a round margin, a rough, opaque appearance, and a white coloration with a dry texture. The colony size was 4 mm in diameter. The characteristics of both colonies under the microscope and on a plate are shown in Figure 4.

Molecular identification

The result revealed that UPMBC1 and UPMBE2 displayed a bright single band on gel electrophoresis. The nucleotide size for both EB was about 1500 bp (Figure 3). Sequences obtained from the sequencing company were compared with the sequence database in GenBank via the National Center for Biotechnology Information (NCBI). From the database, UPMBC1 was identified as *Enterobacter tabaci* (MH794127.1) and UPMBE2 as *Bacillus cereus*, with 99% (NR 146667.2) and 98% (KC 248214.1) nucleotide similarity, respectively. The actual nucleotide size of *E. tabaci* and *B. cereus* was 1405 bp and 1126 bp, respectively.

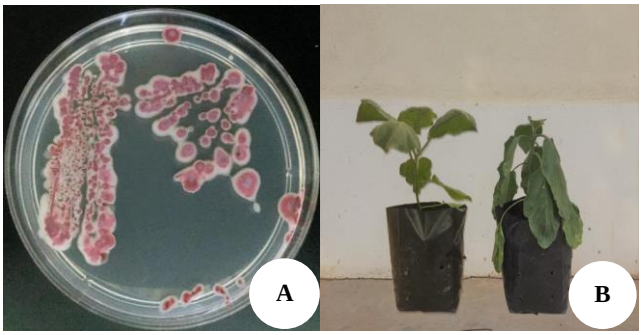

Figure 1. A. Isolated *Ralstonia solanacearum* strains on Kelman's Tetrazolium Chloride (TZC) media. B. The treated eggplant seedling showed wilting symptoms where all the leaves drooped on the third week (*right*) and the control eggplant seedling showed no symptoms of wilting (*left*)

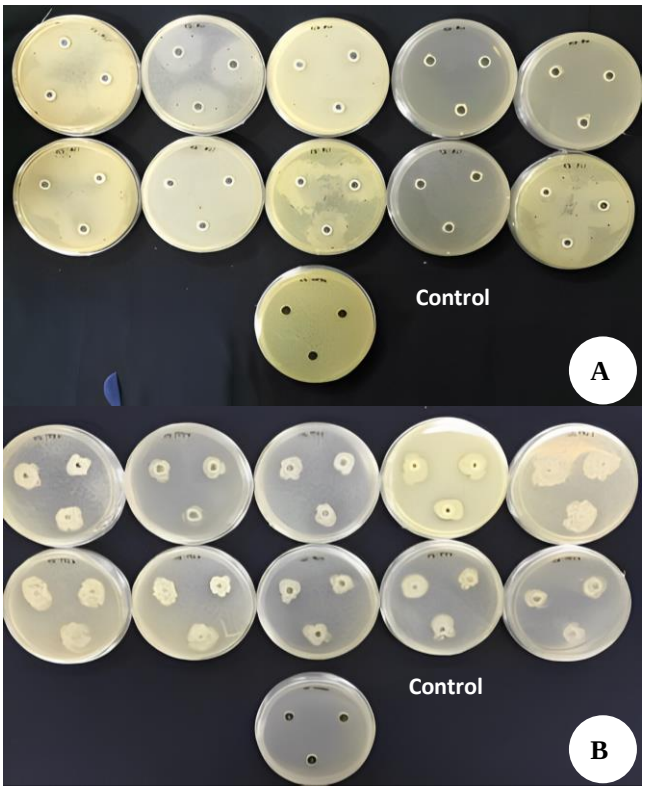

Figure 2. In vitro antagonistic assessment of (A) UPMBC1 and (B) UPMBE2

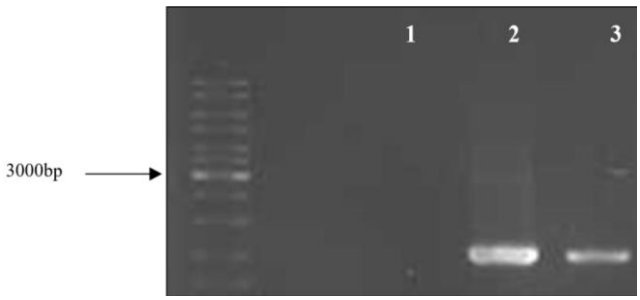

Figure 3. A bright band for *Enterobacter tabaci* (Lane 2) and *Bacillus cereus* (Lane 3) in gel electrophoresis. No band yielded by the control (Lane 1).

Table 1. The Tukey's mean separation analysis of 27 endophytic bacteria against *Ralstonia solanacearum* in the in vitro antagonistic assay

Endophytic bacteria	Radius (mm)	Host plants	States
UPMBC1	14.64^{a}	Chili	Selangor
UPMBE2	12.55^{ab}	Eggplant	Selangor
UPMJT2	9.72^{abc}	Tomato	Johor
UPMBRE4	9.19^{bc}	Eggplant	Selangor
UPMBRE5	8.47^{bcd}	Eggplant	Selangor
UPMAWE2	7.97^{bcde}	Eggplant	Perak
UPMAC3	6.84^{cdef}	Chili	Perak
UPMAC1	6.61^{cdef}	Chili	Perak
UPMJT4	6.51^{cdef}	Tomato	Johor
UPMBC2	6.49^{cdef}	Chili	Selangor
UPMAWE3	5.72^{cdef}	Eggplant	Perak
UPMAC2	5.50^{cdef}	Chili	Perak
UPMNWE3	5.26^{cdef}	Eggplant	Negeri Sembilan
UPMJT3	4.98^{cdef}	Tomato	Johor
UPMPLE2	4.54^{cdef}	Eggplant	Penang
UPMPLE4	4.39^{cdef}	Eggplant	Penang
UPMCLE7	3.57^{def}	Eggplant	Pahang
UPMBRE6	3.53^{def}	Eggplant	Selangor
UPMPC3	3.45^{def}	Chili	Penang
UPMCLE5	3.40^{def}	Eggplant	Pahang
UPMCLE6	3.27^{def}	Eggplant	Pahang
UPMCC1	2.86^{ef}	Chili	Pahang
UPMPLE1	2.72^{ef}	Eggplant	Penang
UPMNWE2	2.49^{f}	Eggplant	Negeri Sembilan
UPMCLE5	2.44^{f}	Eggplant	Pahang
UPMBLE6	2.09^{f}	Eggplant	Selangor
UPMJC4	1.56^{f}	Chili	Johor

Note: The mean separations marked with similar letters are not significantly different according to Tukey's Honest Significant Difference (HSD) test ($P<0.05$)

Discussion

In this study, *R. solanacearum* colonies were isolated from the infected eggplant. Fresh colonies were required because this pathogen could easily lose viability when continuously subcultured on laboratory media (Champoiseau et al. 2009). Moreover, it may also be viable but not culturable if kept at 4°C for an extended period (Milling et al. 2009). From the experiment, three colonies were yielded; however, only one virulent colony of *R. solanacearum* was successfully isolated. The virulent colony was determined by its cultural appearance, and further pathogenicity test was conducted. *R. solanacearum* on culture media showed white color with a pinkish center and watery appearance, whereas in the pathogenicity test, it successfully induced 80% wilting in the tested eggplant. In a previous study, *R. solanacearum* enhanced its biosynthesis of virulence factors by utilizing the host plant metabolites (Shen et al. 2020). The L-glutamic acid acquired from the host plant was proven to be associated with increased Exopolysaccharide (EPS) production, swimming motility, cellulase activity, and biofilm formation of *R. solanacearum*. Besides, the acid compound also helps *R. solanacearum* colonize the root and stem cells, thus accelerating DS. Another study also found that *R. solanacearum* assimilated nitrate to promote its EPS

virulence, enhanced root attachment, and initiated infections (Dalsing and Allen 2014).

The distribution of EB among states varies since each state experiences different rainfall distributions, soil fertility, physiology, chemical, pH, and temperature. Based on the in vitro antagonistic assessment, only 27 isolates could inhibit the growth of *R. solanacearum* effectively and consistently (inhibited the growth of *R. solanacearum* in all replicates). Only two EB were subjected to identification as both were the most efficient at outgrowing the *R. solanacearum* in in vitro antagonistic assessment. 16s rDNA was performed to identify the EB because the colony phenotype cannot be trusted 100% (Sousa et al. 2013). That is because the environment always influences the phenotypic appearance of bacterial characteristics (Young 2007). Cultural and morphological characterizations were carried out to support molecular identification. Therefore, as a Gram-negative bacterium, *E. tabaci's* cell-stained pink safranin color parallels with other Enterobacteriaceae, indicating that the cell wall has a thin peptidoglycan layer.

Even with a thin cell wall layer, the bacterium can withstand the ultimate pH and temperature (Beveridge 1999). Furthermore, it has been reported to withstand three atmospheric pressure (atm) and turgor pressure (Koch 1998). Nevertheless, in the present study, the cocci shape of *E. tabaci* was in contrast to the rod shape of Enterobacteriaceae. This can be explained by the abiotic and biotic stresses from the environment, for instance, salinity, predation, and nutrient competition imposed upon the bacterium (Philippe et al. 2009). Furthermore, numerous studies used *Escherichia coli* as a model in shape-changing studies (Kerksiek 2009; Philippe et al. 2009; Pilizota and Shaevitz 2014). Therefore, the bacterial shape changes from rod to cocci when there are differences in surrounding osmotic pressure. Thus, enduring a harsh environment triggers *E. coli* to reduce its size from rod to cocci. Therefore, the binding protein synthesizing the peptidoglycan is distracted when facing a difficult condition. This distraction disturbed the formation of cell walls and led to a smaller bacterium (Philippe et al. 2009). Therefore, it can be deduced that the odd shape of *E. tabaci* in this study was due to evolution and adaptation from the wild environment the bacterium faces.

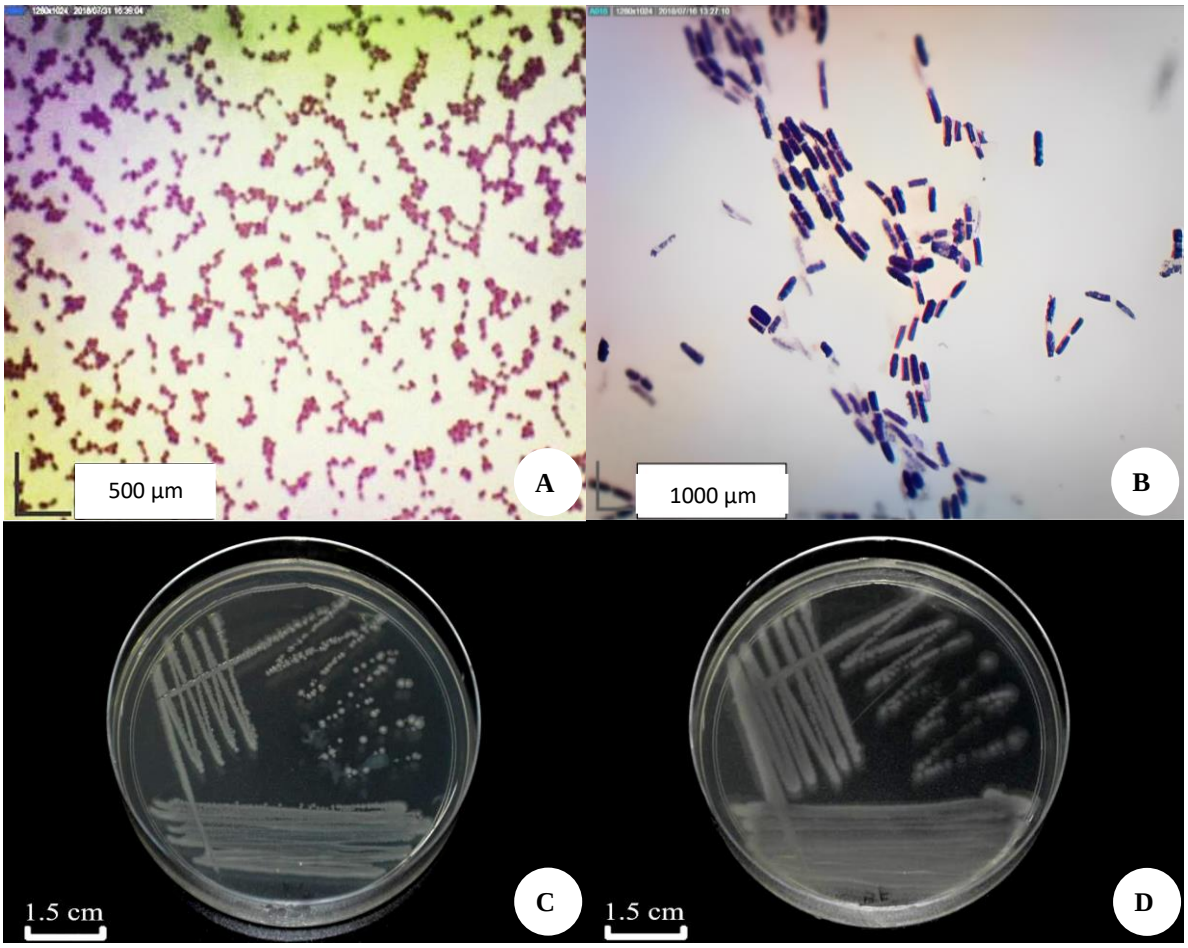

Figure 4. Gram-staining images of endophytic bacteria captured with the Dino-Lite microscope camera (A) *Enterobacter tabaci* with cocci cell shape and stained pink safranin color, (B) *Bacillus cereus* with rod cell shape and stained purple crystal violet color, (C) colony appearance of *Enterobacter tabaci* on nutrient agar and (D) colony of *Bacillus cereus* on nutrient agar

Several studies have been published on the potential Gram-negative bacterium as BCA against *R. solanacearum,* such as *Pseudomonas* sp. Some mechanisms employed by the species are the production of secondary metabolites, enzymes, and antimicrobial compounds to induce the host plant's defense, apart from the ability to expel the pathogen in a nutrient competition physically. *Pseudomonas brassicacearum* was reported to release 2, 4-diacetylphloroglucinol (2,4-DAPG), a siderophore, a protease, and hydrogen cyanide (HCN) to inhibit the growth of *R. solanacearum* (Zhou et al. 2012). A different study documented Pseudomonas strains to stimulate endophytic *Azospira* populations and induced systemic resistance to inhibit *R. solanacearum*. In addition, the plant's defense-related enzymes and defense signaling marker gene expression levels were escalated upon inoculation with *Pseudomonas* strains (Shang et al. 2021). Nonetheless, the induction of systemic resistance was determined to involve the activities of superoxide dismutase, catalase, and polyphenol oxidase (Shang et al. 2021). In another experiment, *Pseudomonas mosselii* triggered tobacco's ethylene- and jasmonate-dependent defense signaling pathways and reduced the DS by 9.1% (Zhuo et al. 2019). Even though Enterobacteriaceae is commonly associated with a pathogen, some of it has the capability of a BCA. Other than *E. tabaci*, *Enterobacter cloacae* were also effective against *R. solanacearum.* The bacterium could suppress and produce some specific antibiotics to induce plant systemic resistance (Mohamed et al. 2020). The *E. cloacae* reduced DS from 93.25% to 10.7% under greenhouse conditions and 26.6% under field conditions, improving crop yield by 20.44%. The BCA-treated potato proved the induction of systemic resistance by the increased: total phenol, salicylic acid contents, and levels of lipoxygenase, peroxidase, and polyphenol oxidase. The *E. cloacae* has also been reported to be effective against RS infection in tomatoes, with 65% biocontrol activity (Xue et al. 2009). Previously, *E. cloacae* were found effective against damping-off disease caused by *Pythium* sp. by producing hydroxamate siderophore aerobactin (Loper et al. 1993). The siderophore production under iron-limited conditions expelled *Phytium* sp. from surviving, thus eliminating the pathogen. Apart from that, *E. cloacae* can also reduce the damping-off incidence by inactivating the stimulatory activities of root exudates. Thus, it could minimize *Phytium ultimum* infection (Kageyama and Nelson 2003). In a different experiment, hyphae of *Pythium aphanidermatum* were deformed and shriveled upon inoculation with *E. cloacae* (Kazerooni et al. 2020).

As for *B. cereus*, the characteristics in this study match those of Bacillaceae. *B. cereus* has a purple-violet stained color, indicating a Gram-positive bacterium. The gram-positive bacterium has a thick peptidoglycan layer, making it more rugged than the Gram-negative. *Bacillus* sp. is one of the famous BCAs and has been widely studied for its potential ability to control phytopathogens, including BW disease. *Bacillus niabensis* and *Bacillus subtilis* produced a halo zone, indicating the production of metabolites against the *R. solanacearum* in vitro (Tuhumury et al. 2021). The antibiosis mechanism is believed to be due to the ability of *B. subtilis* to emit antibiotics, such as Bacillomycin D and iturin. In different studies, *Bacillus velezensis* and *Pseudomonas fluorescens* significantly reduced the population of *R. solanacearum*, thus minimizing the occurrence of BW disease (Elsayed et al. 2020). Both strains enhanced the prokaryotic communities and promoted plant defense against BW disease. *P. fluorescens* was detected attached to the plant's root hairs, lateral roots, epidermal cells, and xylem vessels. Besides *B. subtilis* and *B. velezensis*, *Bacillus thuringiensis* was also used to suppress *R. solanacearum.* Compared to the control, B. thuringiensis cell-free supernatant could suppress BW disease by one-third (Hyakumachi et al. 2013). The Volatile Organic Compounds (VOC) of *Bacillus amyloliquefaciens* significantly suppressed the motility, antioxidant enzymes, EPS production, biofilm production, and root colonization of *R. solanacearum* (Raza et al. 2016). In the experiment, 9 VOCs exhibited 1-11% antibacterial activity against *R. solanacearum*, but the consortium of all VOCs reduced the growth of *R. solanacearum* by 70%. The proteomic study suggested that VOCs of *B. amyloliquefaciens* downregulated *R. solanacearum* protein, which is related to its virulence, antioxidant activity, carbohydrate, protein folding and translation, and amino acid metabolism. Tahir et al. (2017) stated that the VOCs of *B. subtilis* Induced Systemic Resistance (ISR) by significantly increasing Polyphenol Oxidase (PPO) and Phenylalanine (PAL) activities, besides over-expressing the gene related to wilt resistance. In the present study, *E. tabaci* and *B. cereus* significantly inhibited *R. solanacearum* growth in vitro. Further experimentation needs to be conducted to find these strains' mode of action models on *R. solanacearum* growth suppression. The excellent performance of both EB in controlling *R. solanacearum* is an important parameter that may provide new hope in e control of BW disease, particularly *E. tabaci.*

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REFERENCES

Achari GA, Ramesh R. 2014. Diversity, biocontrol, and plant growth promoting abilities of xylem residing bacteria from solanaceous crops. Intl J Microbiol 2014: 296521. DOI: 10.1155/2014/296521.

Artal RB, Gopalakrishnan C, Thippeswamy B. 2012. An efficient inoculation method to screen tomato, brinjal, and chili entries for bacterial wilt resistance. Pest Manage Hortic Ecsyst 18 (1): 70-73.

Bell CR, Dickie GA, Harvey WLG, Chan JWYF. 1995. Endophytic bacteria in grapevine. Can J Microbiol 41 (1): 46-53. DOI: 10.1139/m95-006.

Beveridge TJ. 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. J Bacteriol 181 (16): 4725-4733. DOI: 10.1128/JB.181.16.4725-4733.1999.

Blomme G, Dita M, Jacobsen KS, Vicente LP, Molina A, Ocimati W, Poussier S, Prior P. 2017. Bacterial diseases of bananas and enset: Current state of knowledge and integrated approaches towards

sustainable management. Front Plant Sci 8 (1): 1290. DOI: 10.3389/fpls.2017.01290.

Champoiseau PG, Jones JB, Allen C. 2009. *Ralstonia solanacearum* race 3 biovar 2 causes tropical losses and temperate anxieties. Plant Health Prog 10 (1): 1-11. DOI: 10.1094/PHP-2009-0313-01-RV.

Dalsing BL, Allen C. 2014. Nitrate assimilation contributes to *Ralstonia solanacearum* root attachment, stem colonization, and virulence. J Bacteriol 196 (5): 949-960. DOI: 10.1128/JB.01378-13.

Daunay MC, Janick J. 2007. History and iconography of eggplant. Chron Hortic 47 (3): 16-22.

Egamberdieva D, Wirth SJ, Shurigin VV, Hashem A, Abd Allah EF. 2017. Endophytic bacteria improve plant growth, symbiotic performance of chickpea (*Cicer arietinum* l.) and induce suppression of root rot caused by *Fusarium solani* under salt stress. Front Microbiol 8: 1887. DOI: 10.3389/fmicb.2017.01887.

Elsayed TR, Jacquiod S, Nour EH, Sørensen SJ, Smalla K. 2020. Biocontrol of bacterial wilt disease through complex interaction between tomato plant, antagonists, the indigenous rhizosphere microbiota, and *Ralstonia solanacearum*. Front Microbiol 10: 2835. DOI: 10.3389/fmicb.2019.02835.

Hemelda NM, Safitri R, Suhandono S. 2019. Genetic diversity of *Ralstonia solanacearum*, A phytopathogenic bacterium infecting horticultural plants in Java, Indonesia. Biodiversitas 20: 364-372. DOI: 10.13057/biodiv/d200209.

Hyakumachi M, Nishimura M, Arakawa T, Asano S, Yoshida S, Tsushima S, Takahashi H. 2013. *Bacillus thuringiensis* suppresses bacterial wilt disease caused by *Ralstonia solanacearum* with systemic induction of defense-related gene expression in tomato. Microbes Environ 28 (1): 128-134. DOI: 10.1264/jsme2.me12162.

Jabatan Pertanian. 2021. Booklet Statistik Tanaman. Malaysia.

Kageyama K, Nelson EB. 2003. Differential inactivation of seed exudate stimulation of *Pythium ultimum* sporangium germination by *Enterobacter cloacae* influences biological control efficacy on different plant species. Appl Environ Microbiol 69 (2): 1114-1120. DOI: 10.1128/AEM.69.2.1114-1120.2003.

Kazerooni EA, Al-Shibli H, Nasehi A, Al-Sadi AM. 2020. *Enterobacter cloacae* endofítica exibe atividade antagonista contra a podridão de pepino causada por Pythium. Ciênc Rural 50 (8): e20191035. DOI: 10.1590/0103-8478cr20191035.

Kerksiek K. 2009. Shape matters: Why bacteria care how they look. Infect Res 1 (1): 1-5.

Koch AL. 1998. The biophysics of the Gram-negative periplasmic space. Crit Rev Microbiol 24 (1): 23-59. DOI: 10.1080/10408419891294172.

Liu Y, Kanda A, Yano K, Kiba A, Hikichi Y, Aino M, Kawaguchi A, Mizoguchi S, Nakaho K, Shiomi H, Takikawa Y, Ohnishi K. 2009. Molecular typing of Japanese strains of *Ralstonia solanacearum* in relation to the ability to induce a hypersensitive reaction in tobacco. J Gen Plant Pathol 75 (5): 369-380. DOI: 10.1007/s10327-009-0188-7.

Loper JE, Ishimaru CA, Carnegie SR, Vanavichit A. 1993. Cloning and characterization of aerobactin biosynthesis genes of the biological control agent *Enterobacter cloacae*. Appl Environ Microbiol 59 (12): 4189-4197. DOI: 10.1128/aem.59.12.4189-4197.1993.

Lwin M, Ranamukhaarachchi SL. 2006. Development of biological control *of Ralstonia solanacearum* through antagonistic microbial populations. Intl J Agric Biol 8 (5): 657-660.

Milling A, Meng F, Denny TP, Allen C. 2009. Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of *Ralstonia solanacearum* race 3 biovar 2. Phytopathology 99 (10): 1127-1134. DOI: 10.1094/PHYTO-99-10-1127.

Mohamed BFF, Sallam NMA, Alamri SAM, Abo-Elyousr KAM, Mostafa YS, Hashem M. 2020. Approving the biocontrol method of potato wilt caused by *Ralstonia solanacearum* (Smith) using *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34. Egypt J Biol Pest Control 30 (1): 1-13. DOI: 10.1186/s41938-020-00262-9.

Mondal B, Bhattacharya I, Khatua DC. 2014. Incidence of bacterial wilt disease in West Bengal, India. Acad J Agric Res 2 (6): 139-146. DOI: 10.15413/ajar.2014.0118.

Nair P. 2013. The Agronomy and Economy of Turmeric and Ginger (1st ed). Elsevier, USA. DOI: 10.1016/B978-0-12-394801-4.00001-6.

Philippe N, Pelosi L, Lenski RE, Schneider D. 2009. Evolution of penicillin-binding protein 2 concentration and cell shape during a long-term experiment with *Escherichia coli*. J Bacteriol 191 (3): 909-921. DOI: 10.1128/JB.01419-08.

Pilizota T, Shaevitz JW. 2014. Origins of *Escherichia coli* growth rate and cell shape changes at high external osmolality. Biophys J 107 (8): 1962-1969. DOI: 10.1016/j.bpj.2014.08.025.

Popoola AR, Ganiyu SA, Babalola OA, Ayo-John EI, Fajinmi AA, Kehinde IA, Adegboye TH. 2014. Impact of soil amendments and weather factors on bacterial wilt and yield of two tomato cultivars in Abeokuta, Nigeria. S Afr J Plant Soil 31 (4): 195-201. DOI: 10.1080/02571862.2014.966339.

Raza W, Ling N, Yang L, Huang Q, Shen Q. 2016. Response of tomato wilt pathogen *Ralstonia solanacearum* to the volatile organic compounds produced by a biocontrol strain *Bacillus amyloliquefaciens* SQR-9. Sci Rep 6: 24856. DOI: 10.1038/srep24856.

Scherf JM, Milling A, Allen C. 2010. Moderate temperature fluctuations rapidly reduce the viability of *Ralstonia solanacearum* race 3, biovar 2, in infected geranium, tomato and potato plants. Appl Environ Microbiol 76 (21): 7061-7067. DOI: 10.1128/AEM.01580-10.

Shang XC, Cai X, Zhou Y, Han X, Zhang CS, Ilyas N, Li Y, Zheng Y. 2021. *Pseudomonas* inoculation stimulates endophytic *Azospira* population and induces systemic resistance to bacterial wilt. Front Plant Sci 12: 738611. DOI: 10.3389/fpls.2021.738611.

Shen F, Yin W, Song S, Zhang Z, Ye P, Zhang Y, Zhou J, He F, Li P, Deng Y. 2020. *Ralstonia solanacearum* promotes pathogenicity by utilizing l-glutamic acid from host plants. Mol Plant Pathol 21 (8): 1099-1110. DOI: 10.1111/mpp.12963.

Sousa AM, Machado IM, Nicolau A, Pereira MO. 2013. Improvements on colony morphology identification towards bacterial profiling. J Microbiol Methods 95 (3): 327-335. DOI: 10.1016/j.mimet.2013.09.020.

Stander EIM, Hammes PS, Beyers EA. 2013. Survival of *Ralstonia solanacearum* biovar 2 in soil under different cropping systems. S Afr J Plant Soil 20 (4): 176-179. DOI: 10.1080/02571862.2003.10634931.

Tahat MM, Sijam K. 2010. *Ralstonia solanacearum*: The bacterial wilt causal agent. Asian J Plant Sci 9 (7): 385-393. DOI: 10.3923/ajps.2010.385.393.

Tahir HAS, Gu Q, Wu H, Raza W, Safdar A, Huang Z, Rajer FU, Gao X. 2017. Effect of volatile compounds produced by *Ralstonia solanacearum* on plant growth promoting and systemic resistance inducing potential of *Bacillus* volatiles. BMC Plant Biol 17 (1): 133. DOI: 10.1186/s12870-017-1083-6.

Tankeshwar A. 2015. Gram Staining: Principle, procedure, results. https://microbeonline.com/Gram-staining-principle-procedure-results/.

Thurston HD, Galindo JJ. 1989. Moko del banano y el platano. Enfermedades de Cultivos en el Tropico 1 (1): 125-133.

Toyota K, Kimura M, Kinoshita T. 2000. Microbiological factors affecting the colonization of tomato roots by *Ralstonia solanacearum* YUIRif43lux. Soil Sci Plant Nutr 46 (3): 643-653. DOI: 10.1080/00380768.2000.10409129.

Tuhumury GNC, Hasinu JV, Kesaulya H. 2021. Activity test of *Bacillus* spp against bacterial wilt (*R. solanacearum*) on tomatoes by in vitro. IOP Conf Ser: Earth Environ Sci 883 (1): 012027. DOI: 10.1088/1755-1315/883/1/012027.

Walch G, Knapp M, Rainer G, Peintner U. 2016. Colony-PCR Is a rapid method for DNA amplification of Hyphomycetes. J Fungi 2 (2): 12. DOI: 10.3390/jof2020012.

Winstead NN, Kelman A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42 (11): 628-634.

Xue QY, Chen Y, Li SM, Chen LF, Ding GC, Guo DW, Guo JH. 2009. Evaluation of the strains of *Acinetobacter* and *Enterobacter* as potential biocontrol agents against Ralstonia wilt of tomato. Biol Control 48 (3): 252-258. DOI: 10.1016/j.biocontrol.2008.11.004.

Yadessa GB, van Bruggen AHC, Ocho FL. 2010. Effects of different soil amendments on bacterial wilt caused by *Ralstonia solanacearum* and on the yield of tomato. J Plant Pathol 92 (2): 439-450.

Young KD. 2007. Bacterial morphology: Why have different shapes? Curr Opin Microbiol 10 (6): 596-600. DOI: 10.1016/j.mib.2007.09.009.

Zhou T, Chen D, Li C, Sun Q, Li L, Liu F, Shen Q, Shen B. 2012. Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. Microbiol Res 167 (7): 388-394. DOI: 10.1016/j.micres.2012.01.003.

Zhuo T, Chen S, Fan X, Hu X, Zou H. 2019. An improved control efficacy against tobacco bacterial wilt by an engineered *Pseudomonas mosselii* expressing the ripAA gene from phytopathogenic *Ralstonia solanacearum*. bioRxiv 2019: 510628. DOI: 10.1101/510628.

Zulperi D, Sijam K. 2014. First report of *Ralstonia solanacearum* race 2 biovar 1 causing Moko disease of banana in Malaysia. Plant Dis 98 (2): 275. DOI: 10.1094/PDIS-03-13-0321-PDN.

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Nutritional and biochemical properties of locally produced wine from blended honey and coconut juice

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Abstract. *Tatah VS, Ogodo AC, Boyi RHN, Abah MA, Timothy M, Ayantse LM. 2023. Nutritional and biochemical properties of locally produced wine from blended honey and coconut juice. Asian J Trop Biotechnol 20: 31-36.* Humans have consumed honey since ancient times due to its high medicinal and nutritional value. Coconut (*Cocos nucifera* L.) is highly nutritious, fiber-rich, and contains essential vitamins and minerals. Coconut cream and coconut milk are two of the many edible products made from the coconut fruit. In addition, some wines are produced from a single fruit type or a combination of blended fruits and other raw materials. This research was conducted to determine the nutritional values and biochemical properties of locally produced wine from blended honey/coconut juice. The blended wine in this study was locally prepared and stored for about three years in the refrigerator at about 4ºC. The Association of Official Analytical method was used to determine phytochemical constituents, proximate composition, minerals composition, physicochemical properties, sugar/alcohol contents, and amino acid content. The phytochemical content includes saponins, tannins, flavonoids, glycosides, resins, alkaloids, terpenes, and cardiac glycosides. The wine contains 75.50% moisture, 0.15% carbohydrate, and 0.20% protein. Ash, fat and crude fibers content were 0.14%, 0.25%, and 0.13%, respectively. The essential amino acids in the blended wine were isoleucine, leucine, histidine, lysine, and threonine. And the non-amino acids were glutamine, glutamic acid, serine, alanine, proline, and aspartic acid with high concentrations. Several minerals were also contained in blended wine, i.e., lead, aluminum, calcium, zinc, phosphorus, sulphur, and iron. Several parameters from the blended wine are slightly higher regarding the recommended daily requirement. Therefore, good wine could be produced from blended honey and coconut juice.

Keywords: Coconut juice, fermentation, honey, phytochemicals, proximate composition, wine

INTRODUCTION

Wine is a product of the normal alcoholic fermentation of grape juice. However, the fermented juices of various fruits (apples, berries, peaches, plums, apricots, and herbs) are now also called wine (usually peach wine, etc.).Wine production involves fermentation using yeast, such as (*Saccharomyces cerevisiae)* that convert sugars into alcohol. The definition of wine is an alcoholic beverage derived from fermented grape juice; however, it has been attributed to other alcoholic beverages from fermented fruits and vegetables over the decades (Gupta and Sharma 2009). Fermented beverages and alcoholic drinks are acceptable for consumption, mostly during cultural and social practices, entertainment, customary practices, and religious purposes (Christoph and Bauer-Christoph 2007). Wine production starts with harvesting grapes' juice before fermentation and concludes with various storage and aging steps (Balogu et al. 2016).

Honey is a sweet jelly-like substance (mainly consisting of a monosaccharide of fructose and glucose) made from the nectar of flowers. Honey is a good substance for yeast fermentation due to its high sugar content, to produce alcohol and carbon dioxide gas (Balogu and Towobola 2017). Honey benefits eyes and eyesight (vision), breaks up hard masses, quenches thirst, and balances Kapha. Moreover, it reduces toxicity, stops hiccups, treating bleeding disorders, urinary tract disorders, diabetes, skin diseases, worms, bronchial asthma, cough, diarrhea, nausea, vomiting, and wound healing (Balogu and Towobola 2017; Luvanda and Lyimo 2018). Various alcoholic honey beverages with diverse content of alcohol have been produced from different fermentation processes (Kraus 2017). Most of these processes undertake a minimum of 21 days for fermentation to achieve 7.6%-22% alcohol. Alcoholic beverages of a mixture of honey and fruits have been documented mainly in personal blogs and unpublished studies (Gupta and Sharma 2009).

In the food processing industry, blending is the art of developing different colors, aromas, astringencies, and tastes to suit consumers. Blending could improve the wine's quality, correct deficiencies, enhance complexity, balance flavors, and produce the final product within legal specifications (Lauren et al. 2012). However, the success of blending relies solely on sensory evaluation, and the consumer likes and dislikes the blends if there are high differences in the blended wines (Kovacevic et al. 2003). For example, juice or extract of coconut fruit could be mixed with honey for mead production. Coconut (*Cocos nucifera* L.) belongs to Arcaceae or Palmae; the fruit is characterized by its hard outer shell and white inner flesh (Balogu and Towobola 2017). Coconut water is replaced

by coconut meat and air as the fruit ripens. Coconut milk is a liquid extract of grated and squeezed white coconut flesh rich in fat, minerals, and vitamins (Boateng et al. 2016).

Numerous secondary metabolites in food sources from plants and plant extracts exhibit various bioactivities. They can be used for disease prevention and treatment. For instance, tannins are potential antibacterial and antiviral (Balogu et al. 2016; Tatah et al. 2022). Flavonoids are antibacterial, anti-inflammatory, anti-allergic, anti-mutagenic, antiviral, antineoplastic, anti-thrombotic, and vasodilatory activities. A small number of alkaloids (morphine, codeine, and cocaine) are harmless (Formica and Regelson 1995) and used as painkillers, anesthetics, antimalarial, stimulants, and insecticides (Aiko et al. 2006). Flavonoids also play vital roles in anti-inflammatory, anti-allergic, and anti-cancer functions (Formica and Regelson 1995). Tannins cause protein inactivation; hence they are used as insecticides. They also possess astringent properties. Tannins can inactivate Polio Virus, Herpes simplex, and other enteric viruses (Jaykus 2000). Saponins are natural antibiotics reducing cardiovascular diseases and cholesterol levels. Carotenoids are known for their antioxidant properties, helping the body to destroy free radicals. Carotenoids have equally been shown to possess antihypertensive properties (Khanavi et al. 2013). The present study aims to analyze the biochemical and nutritional quality of wine produced from honey and coconut juice blends.

MATERIALS AND METHODS

Sample collection

A 200 mL of honey/coconut wine was obtained from Federal University Wukari, Taraba State Microbiology Laboratory, Nigeria. The wine sample was locally produced and stored in the refrigerator for about three (3) years at 4ºC.

Phytochemical analysis

Saponins

A 2 mL of the wine sample was weighed into a 125 cm conical flask, added with 100 mL of Isobutyl alcohol, and shaken for 5 hours using an electric shaker. The mixture was then filtered with No 1 Whatman filter paper into a 100 mL beaker containing 20 mL of 40% saturated solution of magnesium carbonate ($MgCO_3$). The mixture obtained was re-filtered again to get a clean, colorless solution. Next, 2 mL of the colorless solution was taken into a 50 mL volumetric flask pipetted and added with 2 mL of 5% iron (iii) chloride ($FeCl_3$) solution and made up to the mark with distilled water. It was allowed to stand for 30 minutes for the color to develop. The absorbance was read using a spectrophotometer against the blank at 380 nm (Brunner et al. 2013).

Cardiac glycosides

A 200 mL of the wine sample was pipetted into a 250 mL conical flask. Chloroform was added and mixed properly using the electric shaker for 1 hour. The mixture was filtered into a 125 cm conical flask. Next, 10 mL of pyridine and 2 mL of 29% sodium nitroprusside were added and shaken thoroughly for 10 min. Finally, 3 mL of 20% NaOH was added to the mixture, and a brownish-yellow color developed. Various concentrations of glycosides standard (Digitoxin) (0-50 mg/mL) were prepared from the stock solution, and the absorbance was read at 510 nm (Newman et al. 2008).

Flavonoids

The aluminum chloride method determined total flavonoid content using catechin as a standard. First, 2 mL of the wine sample and 4 mL of distilled water were appropriately mixed in a 10 mL volumetric flask. Next, 5 mL of the mixture was added with 0.3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminum chloride, and the mixture was then incubated at room temperature for 6 min. Next, 2 mL of 1M sodium hydroxide was added to the reaction mixture; the final volume was immediately made up to 10 mL with distilled water. Finally, the mixture absorbance was measured at 510 nm against a blank using a spectrophotometer (Aiyegoro and Okoh 2010).

Alkaloids

A 2 mL of the wine sample, 5 mL of phosphate Buffer (pH 4.7), and 5 mL BCG solution were appropriately mixed in a 10 mL volumetric flask. Next, the solution was diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against a blank prepared without the sample. Atropine was used as a standard, and the mixture was calculated as the atropine equivalents (Kim et al. 2021).

Tannins

A 2 mL of the wine sample was mixed with 0.5 mL Folin-Ciocalteau's reagent. Next, 1 mL of saturated Na_2CO_2 solution and 8 mL of distilled water were added to the mixture. The reaction mixture was allowed to stand for 30 min at room temperature. The supernatant was obtained by centrifugation. The absorbance was recorded at 725 nm using a UV-visible spectrophotometer. An increasing concentration of the standard tannic acid was prepared, and the absorbance of the various tannic acid concentrations was plotted for a standard graph (Okuda et al. 1989).

Proximate analysis

Moisture content

An aluminum dish was heated in a Carbolite oven at 105ºC for 5 minutes to eliminate any possible moisture; then, the dish was allowed to cool in a desiccator. The weight of the dish was taken and recorded. Next, 5 mL of the wine sample was poured into the dish and weighed. Next, the dish containing the sample was placed in a Cobaltite oven at 105ºC for 24 hours. It was removed, cooled in a desiccator, and weighed (Odebunmi et al. 2010). The weight of the dish containing the dried sample was recorded, and the moisture was then calculated as follows:

Weight of moisture = weight of sample and dish – the weight of dried sample and dish

$$\% \text{ Weight of dried samle} = \frac{\text{Weight of moisture}}{\text{Weight of sample}} \text{ x } 100$$

Dry matter = 100 - % weight of moisture

Fat content

Fat content was analyzed using the method of AOAC (2005). First, 5 mL of the wine sample was collected in a Beaker. Next, the sample was transferred into a thimble and fixed into the machine accordingly. The beaker was filled with about 50 mL petroleum ether and placed under the fixed thimble containing the sample in the extractor chamber. The thimble was then lowered into the aluminum beaker using the adjustment knob and boiled for 10 min. Water tubing was collected. After this, the thimble was raised for another 10 minutes to rinse the extracted fat into the beaker. The condenser tap was closed for 10 minutes to remove residual petroleum ether. The aluminum beaker containing the extracted fat was removed and placed in an oven for 15 minutes to evaporate the remaining petroleum ether. Then, it was cooled in a desiccator and weighed. The fat content was calculated as follows:

Weight of fat = weight of sample and beaker – the weight of empty beaker % weight of fat = ×100

Crude fiber determination

5 mL of the defatted sample was weighed, dispensed into a quick-fit glass, and added with 50 mL of glacial acetic acid. The sample was placed in the heater in the fume cupboard (digestion flask) at about 200ºC-400ºC for 45 mins for proper digestion. After digestion, the sample was filtered thoroughly with a weighed filter paper; then, it was dried in an oven for 24 hours at 100ºC, weighed again, and recorded. The residue was in a weighed crucible and placed in a cooled furnace, then ashing at 580ºC-600ºC for 4-5 hrs. After cooling down, the crucible was placed in a desiccator, weighed, and recorded (Neubert et al. 1940). The fiber content was calculated as follows;

Weight of residue = weight of filter paper + residue – the weight of filter paper Weight of ash = weight of ash + crucible – the weight of the empty crucible

Weight of crude fiber = weight of ash – the weight of residue

Determination of ash content

According to AOAC (2005), the weight of an empty crucible was recorded, and then 5 mL of the sample was added to the crucible and weighed. Ashing sample at 600ºC for 2 hours and cooled in a desiccator. It was then weighed, then the new weight of the crucible plus ash was recorded. The ash content was calculated as follows;

Weight of ash = (weight of crucible + ash) – weight of crucible

$$\% \text{ Weight of ash} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \text{ x } 100$$

Crude protein determination

The Kjeldahl method was used to determine the crude protein content. First, 5 mL of the wine sample was weighed into the micro Kjeldahl digestion flask, and one tablet of Selenium catalyst was added. The mixture was digested on an electrothermal heater until a clear solution was obtained and cooled. The solution of 50 mL was diluted with distilled water. Next, 5 mL of the diluted solution was transferred into the distillation apparatus. Next, 5 mL of 2% boric acid was pipetted into a 100 mL conical flask (the receiver flask) and added with four drops of methyl red indicator. About 50% NaOH was continually added to the digested sample until the solution turned cloudy, which indicated that the solution had become alkaline. Then distillation was carried out into the boric acid solution in the receiver flask with the delivery tube below the acid level. As the distillation was going on, the pink color solution of the receiver flask turned blue, indicating the presence of ammonia. Distillation was continued until the flask's content was about 50 mL, after which the delivery of the condenser was rinsed with distilled water. The resulting solution was titrated with 0.1 M HCl in the conical flask (Aiyegoro and Okoh 2010).

Carbohydrate

The carbohydrate content in the sample was determined by calculation which is as follows;

Weight of carbohydrate = sum of values (protein+ash+fat+phosphorus+fiber+moisture+ calcium)– 100

Mineral composition

The minerals in the wine sample were analyzed using the spectrophotometer, and 2 mL of wine was placed in a 50 mL volumetric flask, added with 2 mL of perchloric acid, 1ml of H_2SO_4, and 5mL of HNO_3. The mixtures were placed in a water bath and evaporated to dryness. The solution was cooled and filtered into a 100 mL standard flask and diluted to volume with distilled water. Therefore, to analyze the minerals separately Atomic absorption spectrophotometer was used.

Physicochemical analysis

pH

The pH meter was calibrated using distilled water before use. Two mL of the wine sample was weighed accurately and dissolved in 25 mL of distilled water in a conical flask. The solution was transferred into a beaker. The pH meter electrode was inserted into the solution in the beaker, and the pH was recorded.

Temperature

The temperature of the wine sample was determined using a laboratory thermometer. Two mL of the wine sample was added with 20 mL of distilled water to a 100 mL beaker, and the thermometer was directly inserted into the solution.

Determination of alcohol content

Exactly 50 mL of the wine was added to a distillation flask and added with NaOH to become alkaline. Next, the samples were distilled until the temperature of the distillate reached 100ºC. Then, the distillates were diluted with distilled water to precisely 50 mL in a pre-weighed volumetric flask. Next, the flask and the liquid were weighed, and the solution density was calculated (Okuda et al. 1989). The alcohol contents were finally obtained using the Density – Alcohol Table.

Sugar

Two (2) mL of the wine sample was placed in a beaker, added with distilled water to mark 100 mL, and 2-3 drops of phenolphthalein were added. Then, NaOH solution was added until it turned pink, followed by adding HCL continuously until the solution turned to its original color. Distilled water was added to the 200 mL and marked (V1). Next, 5 g of Cupric acid was added to 50 mL of the above solution, boiled for 10 min in a water bath, and cooled; then distilled water was added to the 200 mL and marked (V2). Furthermore, to 2 mL of the wine sample, 5 mL of Fehling solution A and B was added and boiled for 2 min and then cooled, after which 2-3 drops of methylene blue were added and titrated with the wine solution of volume marked (V2) above until it turned brick red (Odebunmi et al. 2010).

Total sugar = Fehling solution constant 0.051 × 200 × 200 × 100⁄2 × 50 vol. of the wine solution used for titration.

Amino acid profile analysis

An ion-exchanger chromatography and a colorimeter were used to observe the amino acid composition of blended wine. First, the different amino acids in the wine sample were separated based on their and collected in a beaker by eluding the sample with sodium extract buffer. The amino acid in each beaker was then identified by calculating the buffer volume used in eluding the individual amino acids and the pH of the amino acid, thereby comparing it to the standard. Next, the same volume of each identified amino acid was collected in a test tube, and 1 mL of ninhydrin solution was added. All the tubes were covered with aluminum foil and kept for 15 min in a boiling water bath. After heating, the test tube was removed and cooled in cold water, and 1 mL of 50% ethanol was added and mixed till homogenous. In addition, each amino acid concentration in the tubes is determined using a colorimeter (AOAC 2005).

RESULTS AND DISCUSSION

Qualitative/quantitative phytochemical constituent of locally produced wine from blended honey/coconut juice

Table 1 shows the phytochemical constituent of locally produced wine from blended honey/coconut juice. The result revealed the presence of saponins, tannins, flavonoids, glycosides, resin, alkaloids, terpenes, and Cardiac glycosides. The sample's highest phytochemicals concentration was tannins (3.411 mg/100mL), whereas the lowest was glycosides (1.842 mg/100mL). Saponins, flavonoids, resins, alkaloids, terpenes, and Cardiac glycosides content were 2.892 mg/100mL, 3.262 mg/100mL, 1.921 mg/100mLL 1.961 mg/100mL, 2.471 mg/100mL and 2.492 mg/100mL, respectively.

Proximate composition of locally produced wine from blended honey/coconut juice

The nutritional composition of blended wine is shown in Table 2. The moisture, protein, fat, ash, fiber, and carbohydrates content were 75.50%, 0.23%, 0.25%, 0.14%, 0.13%, and 0.15%, respectively.

Mineral composition (heavy metals) of locally produced wine from blended honey/coconut juice

Table 3 shows the minerals content of the blended wine were lead, aluminum, calcium, sodium, magnesium, zinc, potassium, phosphorus, and iron with an estimated value of 0.01 mg/100mL, 3.20 mg/100mL, 3.20 mg/100mL, 3.80 mg/100mL, 1.80 mg/100mL, 3.40 mg/100mL, 3.2mg/100mL, 2.6 mg/100mL and 0.27 mg/100mL, respectively.

Physicochemical properties of locally produced wine from blended honey/coconut juice

The pH and temperature of locally produced wine were 3.6 and 22°C, respectively (Table 4). The alcohol and sugar content analysis of the blended wine revealed that the wine contained 15.13% alcohol and 2.4% sugar, respectively.

Table 1. Qualitative/quantitative phytochemical constituent of locally produced wine from blended honey/coconut juice

Parameters	Concentration (mg/100mL)	
	QL	QT
Saponins	+	2.892
Tannins	+	3.411
Flavonoids	+	3.262
Glycosides	+	1.842
Resins	+	1.921
Alkaloids	+	1.961
Terpenes	+	2.471
Cardiac glycosides	+	2.492

Note: QL: Qualitative analysis, QT: Quantitative analysis

Table 2. Proximate composition of locally produced wine from blended honey/coconut juice

Parameters	Concentration (%)
Moisture content	75.50
Protein	0.23
Fat	0.25
Ash	0.14
Fiber	0.13
Carbohydrates	0.15

Table 3. Mineral composition (heavy metals) of locally produced wine from blended honey/coconut juice

Parameters	Concentration (mg/100mL)
Lead	0.01
Aluminum	3.20
Calcium	3.20
Sodium	3.80
Magnesium	1.80
Zinc	3.40
Potassium	3.20
Phosphorus	2.60
Iron	0.27

Table 4. Physicochemical properties of locally produced wine from blended honey/coconut juice

Parameters	Values
Temperature (ºC)	21
Sugar content (%)	2.4
Alcohol content (%)	15.13
pH	3.6

Table 5. Amino acid profile of locally produced wine from blended honey/coconut juice.

Parameters	Concentration (mg/100mL)
Asp	131.7±7.5
Glu	121±5.0
Asn	214.1±5.4
Ser	119.8±4.0
Gln	252.0±12.1
His	14.5±0.4
Arg	22.6±0.8
Thr	76.2±9.0
Ala	189±5.2
Val	3.8±0.4
Lys	11.5±0.3
Ile	6.7±0.6
Leu	29.8±1.4
Phe	19.7±1.2
Pro	40.2±1.7

Amino acid profile analysis of locally produced wine from blended honey/coconut juice

The amino acids content of locally produced wine (Table 5) were asparagine, glutamic acid, serine, glutamine, histidine, arginine, threonine, alanine, tyrosine, valine, isoleucine, leucine, phenylalanine, and proline with the value of 131±7.5 mg/100mL, 121.6±5.0 mg/100mL, 117.9±1.4 mg/100mL, 260.4±11.0 mg/100mL, 14.2±0.9 mg/100mL, 22.6±0.4 mg/100mL, 7.47±5.2 mg/100mL, 1.924±5.9 mg/100mL, 34.0±07 mg/100mL, 38±12.1 mg/100mL, 6.1±0.2 mg/100mL, 29.8±1.4 mg/100mL, 19.1±16 mg/100mL and 40±16 mg/100mL, respectively.

Discussion

Honey produced by honey bees (*Apis mellifera* Linnaeus 1758) is a good energy source with antioxidant and antimicrobial properties. The concentrated sugar complex mixture in honey contains some carbohydrates, aromatic substances, waxes, minerals, pollen grains, pigments, and organic and amino acids. Coconut milk is a liquid extract of grated and squeezed white meat of the coconut, which is highly rich in fat, minerals, and vitamins. The phytochemical results of locally produced wine from blended honey/coconut juice contained higher saponins, tannins, flavonoids, and glycosides than that revealed in tetraptera fruits (Neubert et al. 1940). Saponins, tannins, flavonoids, and glycosides content in Tetraptera fruits was 2.892, 3.411, 1.70, and 1.58, respectively (Neubert et al. 1940). The alkaloids in the blended wine were estimated to be 1.961 mg/100mL, lower than that obtained by Ebana et al. (2019). Phytochemicals from the present study revealed that wine has great health benefits. For instance, several alkaloids have been used as painkillers, anesthetics, antimalarial, and stimulants. In addition, flavonoids also play vital roles as anti-inflammatory, anti-allergic, and anti-cancer roles.

Furthermore, flavonoids have been shown to possess antihypertensive properties (Haddad et al. 2008). In addition, Tannins cause protein inactivation, hence used as insecticides, and possess astringent properties. Tannins can inactivate Polio Virus, Herpes simplex, and other enteric viruses. Saponins also serve as natural antibiotics, reducing cardiovascular diseases and cholesterol levels (Haddad et al. 2008).

Proximate analysis results of wine produced from blended honey/coconut had 75.50 mg/100mL moisture content, 0.23 mg/100mL protein, 0.25 mg/100mL fat, 0.14 mg/100mL ash content, 0.13 mg/100mL, and 0.15 mg/100mL carbohydrates. The moisture content of blended wine (75.50%) is lower than that of *poporo* (beverage originating from sorghum stem sheath) (94.60%) (Somogyi 1926). The percentage of ash and carbohydrate obtained from this study are similar to that reported by (Michael and Hans-Werner 1998), which are 0.14%, 0.15% and, 0.12%, 0.20%, respectively.

Several minerals contained in blended wine were lead, sodium, magnesium, zinc, calcium, aluminum, iron, and phosphorus with an estimated values of: 0.01 mg/100mL, 3.80 mg/100mL, 3.40 mg/100mL, 3.20 mg/100mL, 3.20 mg/100mL, 3.20 mg/100mL, 2.60 mg/100mL, 1.5 mg/100mL and 0.27 mg/100mL, respectively. The lowest mineral content was iron, with a value of 0.27 mg/100 mL. Lead in the blended wine (0.01 mg/100mL) may be due to contaminants from the glass wares or equipment used in the analysis. However, this value does not exceed the recommended value. Most of the minerals analyzed from the blended wine are either lower or higher, while others fall within the range compared to those reported in the literature.

The alcohol and sugar content of the blended wine was 15.13% and 2.40%, respectively, while the alcohol concentration obtained by (Ebana et al. 2019) was 14%. The alcohol and sugar contents of the blended honey/coconut wine were 15,13% and 2,4%. These values were higher than blended wine in the study by Markov et al. (2021), with alcohol and sugar content of 9.5% and 0.25%, respectively.

Several amino acids were obtained, for example, aspartate, glutamine, glutamic acid, alanine, and asparagine. The estimated values of the amino acids present were observed to be significantly high, presented as follows: <131.7±7.5, <121±5.0, <252.0±12.1, <189±5.2 and <214.1±5.4 respectively (Table 5). On the other hand, valine and isoleucine, with values of <3.8±0.4 and <6.9±0.6, are lower than the values of other amino acids. The essential amino acids obtained were isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, and histidine with the following values: <6.7±0.6, <29.8±1.4, <11.5±0.3, <19.7±1.2, <76.2±9.0, <3.8±0.4 and <14.5±0.4 respectively (Table 5). At the same time, the non-essential

amino acids obtained are; asparagine, aspartate, serine, alanine, and proline, with the estimated values at <131.7±7.5, <121±5.0, <214.1±5.4, <119.8±4.0, <189±5.2, and <40.2±1.7, respectively. The values of valine and lysine are lower than that obtained by Rutherfurd and Gilani (2009), which were <3.8±0.4, <11.5±0.3, and 10.47, 16.15, respectively. All the amino acid values obtained by Rutherfurd and Gilani (2009) were lower than in this study. The high concentration of amino acids in the blended honey/coconut wine shows that the wine will have great nutritional benefits to consumers. It is because amino acids are used to synthesize proteins which helps to repair worn-out tissues and growth. In addition, proteins also serve as catalytic enzymes in a chemical reaction.

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REFERENCES

Aiko T, Michael JC, Daigo T, Pyoyun P, Barry S. 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell 18 (4): 1052-1066. DOI: 10.3389/FPLS.2017.00275.

Aiyegoro OA, Okoh AI. 2010. Preliminary phytochemical screening and in vitro antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC. BMC Complement Altern Med 10 (1): 1-8. DOI: 10.1186/1472-6882-10-21.

AOAC. 2005. Official Methods of Analysis. 22nd ed. Association of Official Analytical Chemists, Washington, USA. DOI: 10.22069/PSJ.2017.2017.13717.1271.

Balogu TV, Abdulkadir A, Ikegwu MT, Akpadolu B, Akpadolu K. 2016. Production and sensory evaluation of non-alcoholic wine from sugarcane and tiger nut blend using *Saccharomyces cerevisiae*. Intl J BioSci Agric Technol 7: 7-14. DOI: 10.3390/fdermentation3020016.

Balogu TV, Towobola O. 2017. Production and quality analysis of wine from honey and coconut milk blend using *Saccharomyces cerevisiae*. Fermentation 3 (2): 16. DOI: 10.3390/fdermentation3020016.

Boateng L, Ansong R, Owusu WB, Steiner-Asiedu M. 2016. Coconut oil and palm oil's role in nutrition, health and national development: A review. Ghana Med J 50 (3): 189-196. DOI: 10.4314/gmj.v50i3.11.

Brunner G. 2013. Gas Extraction: An Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Processes Volume 4. Springer Science & Business Media, Dordrecht. DOI: 10.1002/bbpc.19961000668.

Christoph N, Bauer-Christoph G. 2007. Flavour of spirit drinks: Raw materials, fer-mentation, distillation, and ageing. In: Berger RG (eds.). Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. Springer Berlin Heidelberg, Heidelberg. DOI: 10.1007/978-3-540-49339-6_10.

Ebana RUB, Edet UO, Anosike KI, Etok CA, Kanu TO. 2019. Nutritional analysis and wine production potentials of *Telfairia occidentalis* (fluted pumpkin) leaves and *Cucumis sativus* L.(cucumber) using Baker's and palm wine yeast strains. World News Nat Scis 22: 12-30. DOI: 10.9734/AJMAH/2016/29362.

Formica JV, Regelson W. 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 33: 1061-1080. DOI: 10.1016/0278-6915(95)00077-1.

Gupta JK, Sharma R. 2009. Production technology and quality characteristics. Food Sci Food Saf 1: 23-27.

Haddad PR, Sterns M, Wardlaw J. 2008. Analysis of Wine - an Undergraduate Project. Australian National University, Australia. DOI: 10.3923/ajft.2014.162.171.

Jaykus L. 2000. Enteric viruses as emerging agents of foodborne disease. Irish J Agric Food Res 39 (2): 245-55. DOI: 10.1128/aem.70.11.6603-6610.2004.

Khanavi MH, Vatandoost NK, Dehaghi AS, Dehkordi MM, Sedaghat A, Hadjiakhoondi A, Hadjiakhoondi F. 2013. Larvicidal activities of some Iranian native plants against the main malaria vector, *Anopheles stephensi*. Acta Med Iranica 2013: 141-147.

Kim HG, Nguyen TN, Lee YG, Lee MH, Lee DY, Lee YH, Baek NI. 2021. New phenylalkanoids from the rhizome of *Cnidium officinalis* Makino. Appl Biol Chem 64 (1): 1-7. DOI: 10.1186/s13765-021-00658-7.

Kovacevic GK, Staver M, Persuric D, Banovic M, Komes D, Gracin L. 2003. Influence of blending on the aroma of Malvasia Istriana wine. Food Technol Biotechnol 4: 305-314.

Kraus MW. 2017. Voice-only communication enhances empathic accuracy. Am Psychol 72 (7): 644-654. DOI: 10.1037/amp0000147.

Lauren DT, Renee T, Threlfall T, Jean-François M. 2012. Optimization of blended wine quality through maximization of consumer liking. Food Qual Pref 24: 1-47. DOI: 10.1016/foodqualL.2011.08.010.

Luvanda FT, Lyimo ME. 2018. Evaluation of antimicrobial and antioxidant attributes of Tanzanian honey from two agroecological areas. Biofarmasi J Nat Prod Biochem 16: 69-82. DOI: 10.13057/biofar/f160203.

Markov AS, Dolgolyuk IV, Nazimova EV, Sergeeva LY. 2021. Investigation of the potential of industrial carrot processing waste for the release of bioactive substances. In IOP Conf Ser: Earth Environ Sci 640 (6): 062030. DOI: 10.1088/1755.1315/640/6/062030.

Michael F, Hans-Werner L. 1998. Hydrolysis and amino acid composition analysis of proteins, J Chromatograph A 826 (2): 109-134. DOI: 10.1016/s0021-9673(98)00721-3.

Neubert AM, Fred V, St. John JL. 1940. Determination of crude fiber. Ind Eng Chem Anal Ed 12 (8): 451-451. DOI: 10.1021/ac50148a004.

Newman RA, Yang P, Pawlus AD, Block KI. 2008. Cardiac glycosides as novel cancer therapeutic agents. Mol Interv 18 (1): 36. DOI: 10.1124/mi.8.1.8.

Odebunmi EO, Oluwaniyi OO, Bashiru MO. 2010. Comparative proximate analysis of some food condiments. J Appl Sci Res 6 (3): 272-274.

Okuda T. Takashi Y, Tsutomu H. 1989. New methods of analyzing tannins. J Nat Prod 52 (1): 1-31. DOI: 10.1021/np50061a001.

Rutherfurd SM, Gilani GS. 2009. Amino acid analysis. Curr Protoc Protein Sci 58 (1): 11-19. DOI: 10.1002/0471140864.ps1109s58.

Somogyi M. 1926. Notes on sugar determination. J Biol Chem 70 (3): 599-612. DOI: 10.1016/S0021-9258(19):50870-5.

Tatah VS, Abu MS, Timothy M, Ogodo AC, Kennedy P. 2022. Nutritional and biochemical analysis of locally produced wine from *Cucumis melo* L. fruit. World J Biol Pharm Health Sci 10 (03): 011-019. DOI: 10.30574/wjbphs.2022.10.3.0084.

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Effects of aqueous extract of polyherbal formulation on acetaminophen-induced hepatotoxicity in albino rats

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Abstract. *Abu MS, Mashi RL, Lawal JY, Nathan F. 2023. Effects of aqueous extract of polyherbal formulation on acetaminophen-induced hepatotoxicity in albino rats. Asian J Trop Biotechnol 20: 37-41.* The present study investigated the hepatoprotective activity of aqueous extract of the polyherbal mixture (*Carica papaya* L., *Allium sativum* L., *Curcuma longa* L., and *Azadirachta indica* A.Juss.) against acetaminophen-induced liver damage in rats. The Wistar albino rats of either sex were divided into every five animals into six groups. They were given the following seven-day treatment, i.e., paracetamol 500 g/kg BW p.o. to induce hepatotoxicity; Silymarin (140 mg/kg BW p.o.) as a reference standard; and three doses of polyherbal extract (100 mg/kg BW p.o., 300 mg/kg BW p.o., and 500 mg/kg BW p.o.). Blood collection by cardiac puncture was carried out after 24 h of the last administration and analyzed for various serum parameters (Serum Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Total Bilirubin (TB), Albumin (ALB), Total Protein (TP), Triglycerides (TGL) and Total Cholesterol (TCHOL). Treatment of the aqueous extract of the polyherbal mixture reduced the elevated levels of AST, ALT, ALP, TB, TGL, and TCHOL. It increased ALB and TP levels to indicate the repair of hepatic damage and demonstrated the aqueous extract hepatoprotective activity of the polyherbal mixture. The aqueous extract of the polyherbal mixture at doses of 100 mg/kg BW, 300 mg/kg BW and 500 mg/kg BW have significant ($p<.005$) effects on the liver of the paracetamol-induced hepatotoxicity rat model.

Keywords: *Allium sativum*, aqueous extract, *Azadirachta indica*, *Carica papaya*, *Curcuma longa*, hepatoprotective, polyherbal

INTRODUCTION

Hepatotoxicity often arises from cellular necrosis associated with oxidative stress generated by elevated free radicals, which can directly cause cell membrane breakdown with consequent changes in metabolic pathways (Sahreen et al. 2011). ROS causes degenerative cellular alterations that impact various main organs, including the heart, liver, lung, and kidney (Pizzino et al. 2017). Furthermore, the injured liver may decrease the antioxidant defense system.

Radiation (e.g., UV radiation, X-rays, etc.), contaminants, and endogenous metabolites produce free radicals in the cells. Large dosages of paracetamol, a frequently used analgesic and antipyretic medication, have been shown in humans and experimental animals to induce hepatotoxicity. Increased lipid peroxidation in the liver is a common characteristic following a hepatotoxic overdose drug (Begum et al. 2022).

According to FAO 2004, more than 6.8 million tons of papaya (*Carica papaya* L.) fruits were produced worldwide on 389,990 hectares of papaya cultivation (Sagadevan et al. 2019). Green papaya fruits, leaves, young shoots, and papaya flowers are consumed as vegetables in Asian nations, while ground dry papaya seeds are used as pepper (Papaya Australia 2017). Papaya also has various therapeutic benefits. Papain, a proteolytic enzyme obtained in milky papaya latex, has several pharmacological and commercial uses (Sharma et al. 2022). For example, Papain is used as an enzymatic debridement for necrotic tissue in burns, ulcers, and other wounds in US FDA-approved topical therapies, as well as the manufacture of vaccines and medications for different digestive illnesses (Sagadevan et al. 2019).

Garlic (*Allium sativum* L.) belongs to the Alliaceae and is the second most extensively used *Allium* after onions. It is cultivated worldwide and utilized as a spice, additive, and medicinal herb (Mazengia et al. 2019; Tesfaye 2021). Garlic contains several sulfur compounds (allicin, diallyl disulfide, S-allylcysteine, and diallyl trisulfide are responsible for their therapeutic benefits. It is eaten raw (fresh leaves or dried cloves) or processed (garlic oil, garlic extracts, and garlic powder), with various chemical compositions and quantities of bioactive components. It has long been recognized as a popular remedy for various ailments and physiological disorders and a useful spice (Shang et al. 2019).

The neem tree (*Azadirachta indica* A.Juss.) has been thought to have excellent health-promoting characteristics for generations (Rudra et al. 2019). Evidence shows that neem has been used to aid healing since 4,500 years ago; Their use originated in ancient India and neighboring countries, where it has long been revered as the most adaptable plant. Even today, the neem tree is recognized as the "Village Pharmacy," with all components having exceptional therapeutic potential.

Curcuma longa L., commonly known as turmeric (Zingiberaceae), is widely recognized in herbal medicine as

a panacea with a broad range of pharmacological properties. Turmeric plant is growing in tropical and subtropical regions worldwide and is widely grown in Asian countries, mainly in China and India. Turmeric may grow up to 1 m tall and has a short stem. Turmeric is an important spice worldwide, with a long history of human use, especially in the East (Nasir et al. 2014). Traditional medicine has used this powder to treat gastrointestinal problems, particularly biliary and hepatic disorders, diabetic wounds, rheumatism, inflammation, sinusitis, anorexia, colitis, and coughs (Ammon et al. 2012).

The study investigated the hepatoprotective activity of aqueous extract of a polyherbal mixture (*C. papaya, A. sativum, C. longa,* and *A. indica*) against acetaminophen-induced liver damage in rats.

MATERIALS AND METHODS

Study area

The study was conducted at the Department of Biochemistry, Federal University Wukari Nigeria, Taraba State, Nigeria, from October 2022 to January 2023.

Sample collection and preparation

Fresh papaya and neem leaves were collected within the Wukari Local Government of Taraba State, then cleaned and dried under shade. Garlic bulbs and turmeric rhizomes were bought in the Wukari market. They were brought to the Federal University Wukari, Biochemistry Laboratory, Nigeria, chopped into smaller pieces, and shade dried under standard laboratory conditions to prevent nutrient loss.

Preparation of crude extracts and polyherbal formulation

Exactly 125 g of each pulverized plant powder was mixed and soaked for 48 hours in 250 mL of distilled water with periodic stirring and mixing. The solution was subsequently filtered through the Whatman filter paper. After filtration, the extract was evaporated and concentrated using a water bath at 99°C. The yield percentage was 15.7%, and the extract was stored at 4°C until further analysis.

Experimental animal

Healthy male Wistar rats of about 120-150 g in weight were used in this study. They were purchased from the animal house of the College of Health Science, Benue State University, Nigeria, and transported to the Animal House of the Department of Biochemistry, Federal University Wukari, Nigeria. They were acclimatized for two weeks and weighed again before starting the experiment (Yakubu et al. 2014).

Animal grouping

Thirty (30) Wistar albino rats were distributed into six groups of five rats in each group. The rats received the following treatment: (i) Group I: Non-paracetamol-induced rats (normal control). (ii) Group II: Paracetamol-induced rats (negative control) 500 mg kg^{-1} BW. (iii) Group III: Paracetamol-induced Nephrotoxic rats treated with 140 mg kg^{-1}BW of silymarin (standard control). (iv) Group IV: Paracetamol-induced Nephrotoxic rats treated with 100 mg extract kg^{-1} BW. (v) Group V: Paracetamol-induced Nephrotoxic rats treated with 300 mg extract kg^{-1} BW. (vi) Group VI: paracetamol-induced Nephrotoxic rats treated with 500 mg extract kg^{-1} BW.

Treatment and induction

Nephrotoxicity was induced by oral administration of paracetamol (500 mg kg^{-1} BW). Paracetamol was dissolved in distilled water, and the paracetamol administration was continued for ten days. After three days of paracetamol induction, each group was given the appropriate treatments, namely administration of the extract of the mixture of *C. papaya, A. indica, C. longa,* and *A. sativum,* and standard drug (silymarin) carried out concomitantly with paracetamol induction for seven days. The rats were fasted for twelve (12) hours, anesthetized using chloroform, and sacrificed at the end of the experimental period. In addition, blood was collected from the heart via cardiac puncture using sterile syringes and needles for further analysis of kidney function.

Parameters for assessing liver function

Remi centrifuge separated serum from the clotted blood at 2,500 rpm for 15 min at 30°C. Serum samples were immediately subjected to biochemical analysis of Serum Aspartate Transaminase (ASP), Serum Alanine Transaminase (ALT), Alkaline Phosphatase (ALP) Total Bilirubin (TB), Total Protein (TP), Albumin (ALB), Total Glycerides (TGL) and Total Cholesterol (TCHOL) by a microplate reader (Power wave XS), using auto-analyzer (DT 60 Ã Chemistry Analyzer).

Statistical analysis

Data from the biochemical analysis were reported as mean ± standard error. One-way ANOVA and Dunnet's test performed the statistical analysis. A 95% confidence interval, $P<0.05$, and $P<0.01$ were considered significant.

RESULTS AND DISCUSSION

Effect of the extract of the polyherbal mixture (*Curcuma longa, Carica papaya, Allium sativum,* and *Azadirachta indica*) on liver function

In the acetaminophen-treated rats, the levels of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP) increased significantly ($p<0.05$) compared to the normal control Group 1 rats in Table 1. Similarly, the levels of Total Bilirubin (TB) increased significantly ($p<0.05$) while that of the Total Protein (TP) and Albumin (ALB) decreased significantly ($p<0.05$) in the acetaminophen-induced but not treated (Group 2) compared to the normal control Group 1 and the standard drug Group 3 as presented in Table 1. Furthermore, the Total Glycerides (TGL) and Total Cholesterol (TCHOL) were significantly ($p<0.05$) increased in the acetaminophen-induced but not

treated Group 2 compared to the normal control Group 1 and the standard drug Group 3 (Figures 1 and 2). However, treatments with the aqueous extract of the polyherbal mixture lowered the elevated levels of AST, ALT, ALP, TB, TGL, and TCHOL and increased the levels of ALB and TP in Groups 4, 5 and 6 when compared with Group 2.

Discussion

This study investigated the hepatoprotective effect of mixed polyherbal aqueous extract on rats induced with acetaminophen. Acetaminophen-induced hepatotoxicity is the most commonly used screening method for testing the hepatoprotective properties of plant extracts. The hepatic damage causes increased serum levels of enzymes, such as Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), and other serum parameters such as Total Bilirubin (TB), Total Glycerides (TGL) and Total Cholesterol (TCHOL). In addition, it indicates cellular damage and loss of functional integrity of the cell membrane of the hepatocytes (Ilukho et al. 2022). The hepatocytes' acetaminophen-induced damage was also observed in impaired protein metabolism, which was indicated by decreased Total Protein (TP) and Albumin (ALB) beyond the normal control group.

The serum ALT, AST, and ALP levels could be used to determine liver function alteration by increasing levels in the cytoplasm of hepatocytes (Giannini et al. 2005). However, they are usually present in low concentrations in the serum under normal physiological conditions; Their functions are unknown and hence are called non-functional enzymes in the serum (Vasudevan et al. 2011). These enzymes leak into the bloodstream while hepatopathy and rupture of cell or organelle membranes occur; therefore, their concentrations are used to determine the damage to liver tissues (Ze et al. 2020).

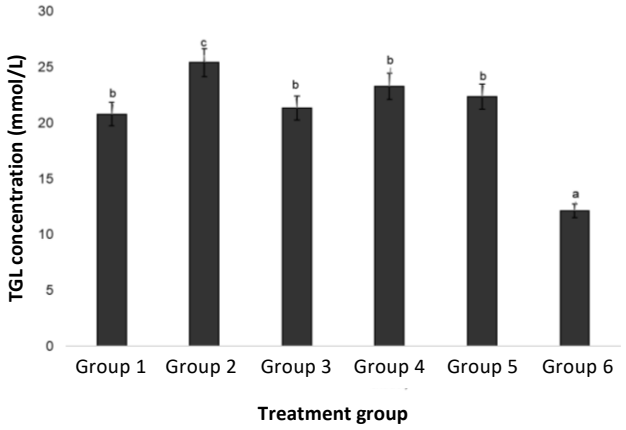

Figure 1. Effect of the administration of the polyherbal extract (*Curcuma longa*, *Carica papaya*, *Allium sativum*, and *Azadirachta indica*) on triacylglycerides levels in Wistar albino rats. N = 5; Result presented as mean ± standard deviation. The same superscripts indicate no significance level, while different alphabets indicate significant differences

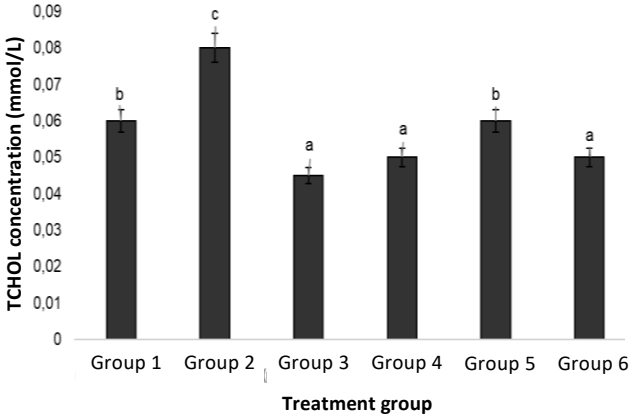

Figure 2. Effect of the administration of the polyherbal extract (*Curcuma longa*, *Carica papaya*, *Allium sativum*, and *Azadirachta indica)* on the Total Cholesterol in Wistar albino rats. N = 5, Result presented as mean ± standard deviation. The same alphabets indicate no significance level, while different alphabets indicate the significance level

Table 1. Effect of polyherbal extracts (*Curcuma longa*, *Carica papaya*, *Allium sativum*, and *Azadirachta indica*) on liver function

Parameter	AST (U/I)	ALT (U/I)	ALP (U/I)	TB (mg/dL)	TP (mg/dL)	ALB(mg/dL)
Group 1	304.82±2.44^{d}	222.14±34.32^{c}	72.57±5.23^{a}	12.11±3.75^{c}	29.16±2.55^{c}	43.97±3.25^{c}
Group 2	364.23±4.49^{e}	339.60±7.00^{d}	348.03±7.20^{f}	21.48±1.56^{d}	19.89±0.33^{b}	34.32±7.93^{b}
Group 3	147.65±7.55^{a}	134.94±6.78^{a}	194.54±10.90^{c}	13.95±3.94^{c}	30.15±1.26^{c}	45.42±0.95^{d}
Group 4	235.30±4.60^{c}	152.67±2.60^{b}	299.54±2.08^{e}	11.62±2.01^{c}	29.36±0.57^{c}	43.60±0.02^{c}
Group 5	178.12±3.89^{b}	156.40±7.87^{b}	162.56±6.55^{b}	7.84±1.32^{b}	29.71±1.61^{c}	44.04±0.88^{c}
Group 6	164.71±5.67^{b}	237.07±6.45^{c}	233.55±7.56^{d}	4.46±0.55^{a}	18.44±1.24^{a}	24.43±1.54^{a}

Note: No = 5; Results are presented as mean ± standard deviation. Results with the same superscripts in the same column indicate no significant difference, while different superscripts indicate significant differences

The increased level of liver enzymes in the bloodstream due to the acetaminophen may be associated with central/submissive liver necrosis, which causes severe hepatic injury (Contreras-Zentella and Hernández-Muñoz 2015). On the other hand, the increased serum ALP may be due to increased synthesis or cholestatic disturbance to the free flow of biliary tract contents due to increased biliary pressure (Wallace 2004). Similarly, increased enzyme levels in rats caused by acetaminophen have been previously reported by Awodele et al. (2016); it was attributed to the damage to the structural integrity of the liver from autolytic breakdown or cellular necrosis.

However, this mixed polyherbal aqueous extract treatment significantly ($p<0.05$) lowered the concentrations of these enzymes as compared to the normal rats and standard drug (silymarin) groups. This finding is similar to Beerendra et al. (2012), in which a polyherbal formulation showed good hepatoprotective activity by lowering the levels of SGOT, alkaline phosphatase, bilirubin parameters, and lipid profiles-cholesterol, triglyceride, and LDL.

Consequently, the hepatoprotective effect of the polyherbal extract may be due to the Phyto-constituents like polyphenols that exhibit varying antioxidant capacities levels (Akachi et al. 2010). Furthermore, the in vitro free radical scavenging activity, such as DPPH, superoxide, and hydroxyl radical scavengings of the polyherbal mixture of various components, suggest the ability of the extract to reduce biological oxidative stress (Madhukiran and Ganga 2011). Hence, the hepatoprotective effect of the extract may be affected by its scavenging free radical activity (Ravikumar and Gnanadesiga 2011).

Bilirubin was significantly increased in the Group 2 experiment (acetaminophen-induced but not treated with extract); however, the high level of bilirubin was lowered significantly ($p<0.05$) in the extract-treated rats (Groups 4, 5 and 6) compared to Group 2 (acetaminophen-induced but not treated). Meanwhile, the bilirubin reduction in Group 4 experiment was comparable with the Groups 1 and 3 experiments that served as normal and standard drug groups respectively while that of Groups 5 and 6 were lower compared to Groups 1 and 3 experiments. Bilirubin is the conventional indicator of liver diseases (Achliya et al. 2004). The bilirubin reduction concentration close to normal in the extract-treated groups may be due to the inhibitory effects on cytochrome P450 or/and the promotion of its glucuronidation (Cavin et al. 2001). On the other hand, it showed that the administration of acetaminophen decreased Total Protein and Albumin levels and increased Total Cholesterol and triglycerides. However, these parameters (total protein, Albumin, Total Cholesterol and triglycerides) were maintained close to normal levels in the extract-treated animals (Groups 4, 5 and 6). Therefore, the aqueous extract of polyherbal treatment indicates inhibitory activity against the adverse effect of acetaminophen. It may result from interfering with cytochrome P450, so the formation of hepatotoxic free radicals could be inhibited. The site-specific oxidative damage in some susceptible amino acids of proteins is now regarded as the primary cause of metabolic dysfunction during pathogenesis which alters the concentration of metabolic products (Bhattacharyya et al. 2014). The normalization of protein, cholesterol, and triglyceride levels in the acetaminophen-induced rats, followed by extract-treated rats, confirms the hepatoprotective effect of the plant extract.

A previous study showed that various parts of the plants (*C. papaya*, *A. sativum*, *C. longa*, and *A. indica*) used in the herbal mixture have potent antioxidant and hepatoprotective properties against paracetamol-induced rats (Chattopadhyay 2003; Somanawat et al. 2013; Awodele et al. 2016; Ghobadi et al. 2019). The findings in this study showed the hepatoprotective effect of these plant extracts, which was observed from the liver enzyme levels approaching normal after plat extract treatment.

In conclusion, the polyherbal used in this study has ameliorative potency against hepatotoxicity-induced rats; therefore, the herbal mixture extract can manage hepatic disorders. The exact mechanism of the hepatoprotection and antioxidation of the polyherbal should be investigated further.

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REFERENCES

Achliya GS, Wadodkar SG, Dorle AK. 2004. Evaluation of hepatoprotective effect of Amalkadi Ghrita against carbon tetrachloride-induced hepatic damage in rats. J Ethnopharmacol 90 (2-3): 229-32. DOI: 10.1016/j.jep.2003.09.037.

Akachi T, Shiina Y, Ohishi Y, Kawaguchi T, Kawagishi H, Morita T, Mori M, Sugiyama K. 2010. Hepatoprotective effects of flavonoids from shekwasha (*Citrus depressa*) against D-galactosamine-induced liver injury in rats. J Nutr Sci Vitaminol 56 (1): 60-7. DOI: 10.3177/jnsv.56.60.

Ammon HP, Anazodo MI, Safayhi H, Dhawan BN, Srimal RC. 2012. Curcumin: A potent inhibitor of leukotriene B4 formation in rat peritoneal polymorph nuclear neutrophils (PMNL). Planta Med 58: 226. DOI: 10.1055/s-2006-961438.

Awodele O, Yemitan O, Ise PU, Ikumawoyi VO. 2016. Modulatory potentials of aqueous leaf and unripe fruit extracts of *Carica papaya* Linn. (Caricaceae) against carbon tetrachloride and acetaminophen-induced hepatotoxicity in rats. J Intercult Ethnopharmacol 5 (1): 27-35. DOI: 10.5455/jice.20160124113528.

Beerendra KS, Dayanandan M, Sunil KM. 2012. Scientific validation of polyherbal hepatoprotective formulation against paracetamol induced toxicity. Asian Pac J Trop Biomed 2 (3). DOI: 10.1016/S2221-1691(12)60488-2.

Begum R, Papia SA, Begum MM, Wang H, Karim R, Sultana R, Das PR, Begum T, Islam MR, Manwar N, Sohanur Rahman MS. 2022. Evaluation of hepatoprotective potential of polyherbal preparations in CCl_4-induced hepatotoxicity in mice. Adv Pharmacol Pharm Sci 2022: 3169500. DOI: 10.1155/2022/3169500.

Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. 2014. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol Rev 94 (2): 329-54. DOI: 10.1152/physrev.00040.2012.

Cavin C, Mace K, Offord EA, Schilter B. 2001. Protective effects of coffee diterpenes against aflatoxin B1-induced genotoxicity: mechanisms in rat and human cells. Food Chem Toxicol 39 (6): 549-56. DOI: 10.1016/s0278-6915(00)00168-x.

Chattopadhyay RR. 2003. Possible mechanism of hepatoprotective activity of *Azadirachta indica* leaf extract: part II. J Ethnopharmacol 89 (2-3): 217-9. DOI: 10.1016/j.jep.2003.08.006.

Contreras-Zentella ML, Hernández-Muñoz R. 2015. Is liver enzyme release really associated with cell necrosis induced by oxidant stress? Oxid Med Cell Longev 2016: 3529149. DOI: 10.1155/2016/3529149.

Ghobadi S, Dastan D, Soleimani M, Nili-Ahmadabadi A. 2019. Hepatoprotective potential and antioxidant activity of *Allium tripedale* in acetaminophen-induced oxidative damage. Res Pharm Sci 14 (6): 488-495. DOI: 10.4103/1735-5362.272535.

Giannini EG, Testa R, Savarino V. 2005. Liver enzyme alteration: A guide for clinicians. CMAJ 172 (3): 367-79. DOI: 10.1503/cmaj.1040752.

Ilukho FA, Fasipe OJ, Aigbe FR. 2022. Evaluating the hepatoprotective, ameliorative and antioxidant potentials of the crude aqueous leafy extracts of *Mangifera indica* plant against acute paracetamol-induced hepatotoxicity in a mouse model. Future Sci OA 8 (6): FSO801. DOI: 10.2144/fsoa-2021-0119.

Madhu KP, Ganga RB. 2011. In vitro evaluation for free radical scavenging activity of methanolic leaf extract of *Cyathea gigantea* (Wall. Ex Hook.). Intl J Pharm Res Dev 3 (2): 95-96.

Mazengia E, Beyene T, Tsegay BA. 2019. Short Communication: Diversity of medicinal plants used to treat human ailments in rural Bahir Dar, Ethiopia. Asian J For 3: 75-82. DOI: 10.13057/asianjfor/r030205.

Nasir H, Sahinfard N, Rafieian M, Rafieian S, Shirzad M, Rafieian-kopaei M. 2014. Turmeric: A spice with multifunctional medicinal properties. J Herb Med Pharmacol 3 (1): 5-8.

Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, Squadrito F, Altavilla D, Bitto A. 2017. Oxidative stress: harms and benefits for human health. Oxid Med Cell Longev 2017: 8416763. DOI: 10.1155/2017/8416763.

Ravikumar S, Gnanadesigan M. 2011. Hepatoprotective and antioxidant activity of a mangrove plant *Lumnitzera racemosa*. Asian Pac J Trop Biomed 1 (5): 348-52. DOI: 10.1016/S2221-1691(11)60078-6.

Rudra PG, Gangawane K, Sucheta GG. 2019. Neem the wonder herb: A short review. Intl J Trend Sci Res Dev 3 (3): 962-967. DOI: 10.31142/ijtsrd23038.

Sagadevan P, Selvakumar S, Raghunath M et al. 2019. Medicinal properties of *Carica papaya* Linn: review. Madridge J Nov Drug Res 3 (1): 120-125. DOI: 10.18689/mjndr-1000118.

Sahreen S. Khan MR, Khan RA. 2011. Hepatoprotective effects of methanol extract of *Carissa opaca* leaves on CCl_4-induced damage in rat. BMC Complement Altern Med 11: 48. DOI: 10.1186/1472-6882-11-48.

Shang A, Cao SY, Xu XY, Gan RY, Tang RY, Corke H, Mavumengwana V, Li HB. 2019. Bioactive Compounds and biological functions of garlic (*Allium sativum* L.). Foods 8 (7): 246. DOI: 10.3390/foods8070246.

Sharma A, Sharma, R Sharma M, Kumar M, Barbhai MD, Lorenzo JM, Sharma S, Mekhemar M. 2022. *Carica papaya* L. leaves: Deciphering its antioxidant bioactives, biological activities, innovative products, and safety aspects. Oxid Med Cell Longev 2022: 2451733. DOI: 10.1155/2022/2451733.

Somanawat K, Thong-Ngam D, Klaikeaw N. 2013. Curcumin attenuated paracetamol overdose induced hepatitis. World J Gastroenterol 19 (12): 1962-7. DOI: 10.3748/wjg.v19.i12.1962.

Tesfaye A. 2021. Revealing the therapeutic uses of garlic (*Allium sativum*) and its potential for drug. Discov Sci World J 30: 8817288. DOI: 10.1155/2021/8817288.

Vasudevan DM, Sreekumar S, Kanan V. 2011. Biochemistry for Medical Students. 6th Edition. Jaypee Brothers Medical Publishers Limited, Trivandrum.

Wallace JL. 2004. Acetaminophen hepatotoxicity: NO to the rescue. Br J Pharmacol 143 (1): 1-2. DOI: 10.1038/sj.bjp.0705781.

Yakubu OE, Nwodo OFC, Joshua PE, Ugwu MN, Odu AD,Okwo F. 2014. Fractionation and determination of total antioxidant capacity, total phenolic and total flavonoids contents of aqueous, ethanol and n-hexane extracts of Vitex doniana leaves. Afr J Biotechnol 13: 693-698. DOI: 10.5897/AJB2013.13225.

Ze C, Ruifeng T, Zhigang S, Jingjing C, Hongliang L. 2020. Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. Free Radic Biol Med 152: 116-141. DOI: 10.1016/j.freeradbiomed.2020.02.025.