Asian Journal of Tropical Biotechnology

| Asian J Trop Biotechnol | vol. 19 | no. 1 | June 2022 |
| ISSN 2775-300X | E-ISSN 2775-4715 |

Capsicum annuum photo by Endah Narwastuti

Asian Journal of Tropical Biotechnology

| Asian J Trop Biotechnol | vol. 19 | no. 1 | June 2022 |

Published semiannually **PRINTED IN INDONESIA**

P-ISSN 2775-300X
E-ISSN 2775-4715

9 772775 471378

Asian Journal of Tropical Biotechnology

| Asian J Trop Biotechnol | vol. 19 | no. 1 | June 2022|

ONLINE
http://smujo.id/bbs

p-ISSN
2775-300X

e-ISSN
2775-4715

PUBLISHER
Society for Indonesian Biodiversity

CO-PUBLISHER
Universitas Sebelas Maret, Surakarta, Indonesia

OFFICE ADDRESS
Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./Fax.: +62-271-663375, email: editors@smujo.id

PERIOD OF ISSUANCE
June, December

Society for Indonesian Biodiversity

Universitas Sebelas Maret Surakarta, Indonesia

Published by Smujo International for The Society for Indonesian Biodiversity and Sebelas Maret University, Surakarta, Indonesia

GUIDANCE FOR AUTHORS

Aims and Scope ***Asian Journal of Tropical Biotechnology*** (**Asian J Trop Biotechnol**)(*formerly Bioteknologi, Biotechnological Studies*) encourages submission of manuscripts dealing with all aspects of biotechnology, especially from tropical regions, that emphasize issues germane to improve human life, includes cell biochemistry, enzymes and proteins, applied genetics and molecular biotechnology, genomics and proteomics, applied microbiology and cell physiology, environmental biotechnology, improvement of crop productivity, public health and waste management, as as well as product formation and manipulation of biological processes for use in the agricultural, pharmaceutical biotechnology and allied industries.

Article types The journal seeks original full-length: (i) **Research papers**, (ii) **Reviews**, and (iii) **Short communications**. Original research manuscripts are limited to 8,000 words (including tables and figures) or proportional to articles in this publication number. Review articles are also limited to 8,000 words, while Short communications should be less than 2,500 words, except for pre-study.

Submission: The journal only accepts online submissions through the open journal system **(https://smujo.id/bbs/about/submissions)** or, for login problems, email the editors at unsjournals@gmail.com (or editors@smujo.id). Submitted manuscripts should be the original works of the author(s). Please ensure that the manuscript is submitted using the template, which can be found at (https://biodiversitas.mipa.uns.ac.id/D/template.doc). The manuscript must be accompanied by a cover letter containing the article title, the first name and last name of all the authors, and a paragraph describing the claimed novelty of the findings versus current knowledge. Please also provide a list of five potential reviewers in your cover letter. They should come from outside your institution and better from three different countries. Submission of a manuscript implies the submitted work has not been published (except as part of a thesis or report, or abstract) and is not being considered for publication elsewhere. When a group writes a manuscript, all authors should read and approve the final version of the submitted manuscript and its revision; and agree on the submission of manuscripts for this journal. All authors should have made substantial contributions to the concept and design of the research, acquisition of the data and its analysis, drafting the manuscript, and correcting the revision. All authors must be responsible for the work's quality, accuracy, and ethics.

Ethics Author(s) must be obedient to the law and/or ethics in treating the object of research and pay attention to the legality of material sources and intellectual property rights.

Copyright If the manuscript is accepted for publication, the author(s) still hold the copyright and retain publishing rights without restrictions. For the new invention, authors must manage its patent before publication.

Open Access The journal is committed to free-open access that does not charge readers or their institutions for access. Readers are entitled to read, download, copy, distribute, print, search, or link to the full texts of articles, as long as not for commercial purposes. The license type is CC-BY-NC-SA.

Acceptance Only articles written in US English are accepted for publication. Manuscripts will be reviewed by editors and invited reviewers (double-blind review) according to their disciplines. Authors will generally be notified of acceptance, rejection, or need for revision within 1 to 2 months of receipt. Manuscripts will be rejected if the content does not align with the journal scope, does not meet the standard quality, is in an inappropriate format, or contains complicated grammar, dishonesty (i.e., plagiarism, duplicate publications, fabrication of data, citations manipulation, etc.), or ignoring correspondence in three months. The primary criteria for publication are scientific quality and significance. **Uncorrected proofs** will be sent to the corresponding author by system or email as .doc or .docx files for checking and correcting typographical errors. The corrected proofs should be returned in 7 days to avoid publication delays. The accepted papers will be published online in chronological order at any time but printed at the end of each month.

Free of charge This publication is dedicated entirely to the advancement of science and technology, therefore author(s), or author institution(s) are not subject to publication fees. **Reprints** The sample journal reprint is only available by special request. Additional copies may be purchased when ordering by email and sending back the uncorrected proofs.

Manuscript preparation Manuscript is typed on A4 (210x297 mm^2) paper size, in a single column, single space, 10-point (10 pt) Times New Roman font. The margin text is 3 cm from the top, 2 cm from the bottom, and 1.8 cm from the left and right. Smaller lettering sizes can be applied in presenting tables and figures (9 pt). Word processing program or additional software can be used; however, it must be PC compatible, use the template, and be Microsoft Word based (.doc or .rtf; not .docx). **Scientific names** of species (incl. subspecies, variety, etc.) should be written in italics, except in italicized sentences. Scientific names (genus, species, author) and cultivar or strain should be mentioned completely for the first time mentioning it in the body text, especially for taxonomic manuscripts. The genus name can be shortened after the first mention, except in early sentences, or where this may generate confusion; name of the author can be eliminated after the first mention. For example, *Rhizopus oryzae* L. UICC 524 can be written hereinafter as *R. oryzae* UICC 524. Using trivial names should be avoided. **Biochemical and chemical nomenclature** should follow the order of the IUPAC-IUB. For DNA sequences, it is better to use Courier New font. Standard chemical abbreviations can be applied for common and clear used, for example, completely written butilic hydroxyl toluene (BHT) to be BHT hereinafter. **Metric measurements** should use IS denominations, and other systems should use equivalent values with the denomination of IS mentioned first. A dot should not follow abbreviations like g, mg, mL, etc. Minus index (m^{-2}, L^{-1}, h^{-1}) suggested being used, except in things like "per-plant" or "per-plot." **Mathematical equations** can be written down in one column with text; in that case, they can be written separately. **Numbers** one to ten are written in words, except if it relates to measurement, while values above them are written in number, except in early sentences. The fraction should be expressed in decimal. In the text, it should be used "%" rather than "percent." Avoid expressing ideas with complicated sentences and verbiage/phrasing, and use efficient and effective sentences.

The title of the article should be written in compact, clear, and informative sentence, preferably not more than 20 words. Name of author(s) should be completely written, especially for the first and the last name. **Name and institution** address should also be completely written with street name and number (location), postal code, telephone number, facsimile number, and email address. We choose local names in Bahasa Indonesia for universities in Indonesia. The mention of "strata" program, should be avoided. Manuscript written by a group, author for correspondence along with address is required (marked with "♥"). **The title page** (first page) should include title of the article, full name(s), institution(s) and address(es) of the author(s); the corresponding authors detailed postage and e-mail addresses (P), and phone (O) and fax numbers (O).

Abstract A concise abstract is required (about 200 words). The abstract should be informative and state briefly the aim of the research, the principal results and major conclusions. An abstract is often presented separately from the article, thus it must be able to stand alone (completely self-explanatory). References should not be cited, but if essential, then cite the author(s) and year(s). Abbreviations should be avoided, but if essential, they must be defined at their first mention. **Keywords** are about five words, covering scientific and local name (if any), research themes, and special methods used; and sorted from A to Z. **Abbreviations** (if any): All important abbreviations must be defined at their first mention there. **Running title** is about five words.

Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

Figures and Tables of a maximum of three pages should be clearly presented. The title of a picture is written down below the picture, while the title of a table is written above the table. Colored figures can only be accepted if the information in the manuscript can lose without those images; the chart is preferred to use black and white images. The author could consign any picture or photo for the front cover, although it does not print in the manuscript. All images property of others should be mentioned the source. Author is suggested referring to Wikipedia for international boundaries and Google Earth for satellite imagery. If not specifically mentioned, it is assumed to refer to these sources. **There is no appendix**, all data or data analysis is incorporated into Results and Discussions. For broad data, it can be displayed on the website as a supplement.

References Preferably 80% of it comes from scientific journals published in the last 10 years. In the text, give the author names followed by the year of publication and arrange from oldest to newest and from A to Z; in citing an article written by two authors, both of them should be mentioned; however, for three and more authors only the first author is mentioned followed by et al. For example, Saharjo and Nurhayati (2006) or (Boonkerd 2003a, b, c; Sugiyarto 2004; El-Bana and Nijs 2005; Balagadde et al. 2008; Webb et al. 2008). Extent citation should be avoided, as shown with the word "cit." Reference to unpublished data and personal communication should not appear in the list but should be cited in the text only (e.g., Rifai MA 2007, pers. com. (personal communication); Setyawan AD 2007, unpublished data). In the reference list, the references should be listed in alphabetical order. Names of journals should be abbreviated. Always use the standard abbreviation of a journal's name according to the **ISSN List of Title Word Abbreviations** (www.issn.org/2-22661-LTWA-online.php). Please include DOI links for journal papers. The following examples are for guidance.

Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158. DOI: 10.13057/biodiv/d070213.

The usage of "et al." in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

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.

THIS PAGE INTENTIONALLY LEFT BLANK

ASIAN J TROP BIOTECHNOL
Volume 19, Number 1, June 2022
Pages: 1-6

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c190101

Effect of seed priming using KCl on the growth and proline accumulation of paprika (*Capsicum annuum*) growing at different water availability

SOLICHATUN♥, TISANY ADIRA PUTRI, WIDYA MUDYANTINI, ARI PITOYO
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax. +62-271-663375, ♥email: solichatun@staff.uns.ac.id

Manuscript received: 22 November 2021. Revision accepted: 11 March 2022.

Abstract. *Solichatun, Putri TA, Mudyantini W, Pitoyo A. 2022. Effect of seed priming using KCl on the growth and proline accumulation of paprika (*Capsicum annuum*) growing at different water availability. Asian J Trop Biotechnol 19: 1-6.* Paprika (*Capsicum annuum* L.) is a vegetable commodity that has high economic value. Paprika cultivation still faces problems related to drought conditions. One technique to increase plant resistance to drought stress is the seed priming technique. Seed priming treatment can use various solutions, including KCl. The purpose of this study was to determine the effect of seed priming with KCl solution on the growth of paprika and accumulation of proline. This research is experimental, using a completely randomized design (CRD) with a combination of seed priming treatment and water availability. Seed priming treatment was variation of concentration of KCl solution of 10, 20, and 40 ppm. The treatment of variations in water availability was 100% field capacity (FC), 75% FC and 50% FC. The experiment used 3 replications. Seed priming treatment using KCL at various concentrations (10, 20, and 40 ppm) significantly affected the growth of paprika (*C. annuum*) that grow on variations in water availability. Seed priming treatment using KCl can increase the resistance of paprika plants to moderate drought conditions. Paprika resistance to drought is characterized by accumulation of proline and regulation of root to shoot ratio.

Keywords: KCl, paprika, proline, seed priming, water availability

INTRODUCTION

Paprika (*Capsicum annuum* L.) is one of the chili varieties grown in Indonesia. In general, the success of paprika and chili cultivation is still constrained by several factors. These factors include the presence of disease and environmental stress. Water availability is one of the environmental factors that cause stress for plants. Water stress occurs when the availability of water in the environment is not at an optimal level. Water stress can be in the form of excess water (flooding) or lack of water (drought).

Drought is considered as one of the most destructive abiotic stresses across the world and creates a huge impact on crop production (Marthandan et al. 2020; Zhu et al. 2021). Drought is an environmental condition that very often inhibits chili growth. Drought stress causes low productivity (Nawiri et al. 2017; Begna 2020). The long dry season is one of the causes of drought in agricultural land. Drought stress can have a negative impact on chili growth. Reducing water availability can cause vegetative growth such as plant height, root length, root dry weight, plant dry weight decreases. The low availability of water also causes the generative growth of chili to be inhibited. The results of the research by Yusniwati et al. (2008) showed that drought stress treatment on several varieties of chili can reduce chili production (Tit Super 29.2%, Jati Profit 47.72%, Hot Chilli 25.74%, Laris 52.63%, and Prabu 50.83 %). According to Matiu et al. (2017), globally, drought has reduced the production of several other horticultural crops, namely corn (11.6%), wheat (9.2%), and soybeans (33.1-12.2%). Drought stress can affect plant growth and production.

Physiologically, plant tolerance to drought is carried out by accumulating proline compounds that act as osmoregulatory and osmoprotectant compounds. Under drought stress conditions, there was an increase in the percentage of leaf proline content compared to normal conditions. Based on the research results of Yusniwati et al. (2008) the leaf proline content in some chili genotypes increased by 12.62% to 646.31%. Drought conditions can also cause metabolic activity disorders in plants due to a decrease in chlorophyll content. Based on research conducted by Armita et al. (2017), the content of chlorophyll a, chlorophyll b and total leaf chlorophyll of control plants were higher than plants that grew under drought stress conditions.

The growth and development of seedlings are one of important stages in plant cultivation. The growth and development of seedlings determine growth at later stages in the plant life cycle. The problem faced in the growth phase of cayenne pepper seedlings is the very fast vegetative growth of the aerial parts. According to de Rezende et al. (2017), growth imbalance results in the formation of fragile and elongated seedlings, with smaller hypocotyl diameters, producing fewer roots, and having a higher sensitivity to biotic and abiotic stresses. These factors can cause a decrease in the ability to grow and

develop seedlings. Uniform seed germination and good and healthy seedling development are key factors for increasing agricultural production.

Seed priming is an alternative technology to overcome seed quality by giving certain treatments to seeds before planting. Seed priming is defined as the preparation of seeds using a variety of procedures in order to increase seed germination rate, percentage germination, and seedling emergence uniformity by manipulating the amount of water available in the seed. The pretreatment starts the germination process but prevents radicle protrusion, after which the seeds are dried until needed. Seed priming treatment can use various solutions, namely polyethylene glycol (PEG), mannitol, sorbitol, glycerol, organic salts such as NaCl, KCl, KNO_3, $MgSO_4$, $CaCl_2$, vermiculite, activated charcoal, clay; or hormones and growth regulators (PGR) such as paclobutrazol, abscisic acid, IAA, gibberellins, kinetin, polyamines and salicylic acid (Mirmazloum et al. 2020).

Plant tolerance to drought stress is controlled genetically and is expressed phenotypically through morphological and physiological adaptations. Based on the research of Naz et al. (2014), KCl treatment in *Pisum sativum* L. seeds can increase root elongation. The treatment of soaking seeds in 2.5% (w/v) KCl solution can induce drought resistance in wheat seeds (*Triticum aestivum* L.) (Eivazi 2012). Considering the economic importance of paprika and also for the development of its cultivation techniques, research on seed priming using KCl on paprika seeds needs to be done. This study aims to determine the effect of seed priming using KCl on the growth and accumulation of proline paprika (*C. annuum*) grown in various water availability.

MATERIALS AND METHODS

Paprika seeds used were taken from ripe fruit. The fruit was obtained from paprika farmers in the Cepogo area, Boyolali, Central Java, Indonesia, harvest year 2020. Growth and germination tests were carried out at the Biology Laboratory of Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret (UNS), and the greenhouse of *Unit Pelaksana Teknis* (UPT) Central Laboratory of UNS, Surakarta, Central Java, Indonesia.

The materials used for seed priming are KCl and aquades. KCl was dissolved in distilled water according to the concentration variations of 10, 20, and 40 ppm. The materials used for the seed viability test were 2,3,5-triphenyltetrazoliumchloride, KH_2PO_4, $Na_2HPO_4.2H_2O$, and aquades. The media used for the growth test was regosol soil that was given compost with a ratio of 2:1. Chemicals to determine proline levels are standard proline, 3% sulfosalicylic acid, ninhydrin acid, toluene, and glacial acetic acid (AAG).

Seed preparation

The seeds are removed from the paprika fruits, then air-dried for 1 week. Dried seeds were used for testing. The seeds used are selected in good condition and uniform (shape, size and color).

Seed viability test

Seed viability assay was done by tetrazolium-based method. For tetrazolium staining, 100 seeds were put in a wet paper at 20°C for 18 hours. Seeds were then wounded by cutting the testa between the radicle and the cotyledon. Wounded seeds were immersed in 1% tetrazolium chloride solution (in phosphate buffer) for 6 hours at 30°C, in dark conditions (ISTA 2016). Seeds were cut in half, observed, and counted the viability percentage based on red color of seed.

The percentage of seed viability is calculated by the following formula:

$$\text{Seed viability (\%)} = \frac{\text{number of viable seeds}}{\text{total number of seeds}} \times 100\%$$

Seed priming treatment using KCl solution at various concentrations

KCl solution was applied as seed priming at concentrations of 10 ppm, 20 ppm and 40 ppm. Paprika seeds are soaked in the solution for 24 hours. After that, the seeds are drained and air-dried at room temperature for 24 hours. The dried seeds will be used for the growth test.

The growth test using different water availability

The water availability test was carried out in polybags filled with soil. The size of the polybag used is 10 x 20 cm. The soil used was regosol type with a pH of 6.0. Each polybag is filled 1000 gram regosol soil. Three seeds were sown per polybag at the depth of 3 cm. The polybags were placed in the greenhouse. The research design used was complete randomized design with 5 replications. The treatment under study is as follows :

100% field capacity = 500 mL of water volume
75% field capacity = 375 mL of water volume
50% field capacity = 250 mL of water volume

Determination of the field capacity of the soil using the gravimetric method. One kilogram of wind-dried soil is weighed and put into a polybag that has been provided with drainage holes. Water is sprinkled on the ground, and left until the soil is evenly wet and water drips from the drainage hole. The soil is left for 6 hours (until the water is no longer dripping). This condition is a condition of 100% soil capacity. The soil is then weighed again, and the difference between its weight and the initial weight of the soil (dry soil) is calculated. The figure obtained is the volume of water that must be added to reach the specified field capacity. The water content of the media is checked every day, and given water so that it is at the specified water content. The data for the plant height, leaf area, fresh weight, root to shoot ratio (fresh weight of root biomass divided by fresh weight of shoot biomass), chlorophyll content, carotenoid content and also proline content was recorded after 2 months of sowing (Kurniawan et al. 2010; Sayyari and Ghanbari 2012; Gayathri et al. (2016).

Shoot: root ratio determined based on fresh weight, according to Kakanga et al. (2017). Chlorophyll and carotenoid content were determined using spectrophotometer (Hendry and Grime 1993). Leaf proline levels were calculated at the end of the growing season using spectrophotometer according to Bates et al. (1973). Samples of fresh leaves (2nd leaf from the tip of the plant) weighing 0.1 g were cleaned and crushed, then extracted with 5 mL of 3% sulfosalicylic acid. After homogenization, 2 mL of the sample was taken and added with 2 mL of 0.14 M ninhydrin acid and 2 mL of glacial acetic acid (AAG) then water bathed at 100°C for 1 hour. The tube containing the sample was put into a beaker containing ice cubes for 5 minutes. The sample was extracted again with 4 mL of toluene and shaken for 15-20 seconds. After shaking it will form 2 layers of liquid. The pink layer was taken and the absorbance was calculated by means of a UV-visible spectrophotometer at a wavelength of 520 nm. The standard solution used is L-proline. There are 5 levels of proline concentration as a standard solution, namely 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M, and is calculated based on the standard curve.

Data analysis

Quantitative data were analyzed by ANOVA (Analysis of Variance) and if there was a significant difference between the treatment groups, further tests were carried out using Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

RESULTS AND DISCUSSION

Seed viability test

The tetrazolium test was carried out at the beginning of the study to determine the viability of the seeds to be used. The results of the tetrazolium test showed that the seeds used had a viability of 100% (Figure 1).

Growth parameters

Growth parameters observed in this study were plant height, leaf area, fresh weight, and shoot:root ratio, presented in Table 1. The results of the analysis of variance showed that combination of seed priming treatment using KCl and water availability had a significant effect on the height of paprika seedlings (Table 1).

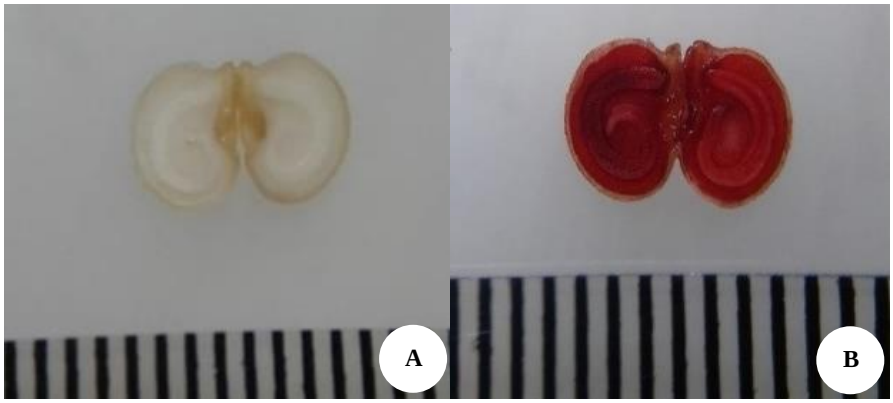


Figure 1. A cross-section of paprika (*Capsicum annuum*) seeds. A. Before soaking pepper seeds in 1% tetrazolium solution, B. After paprika seeds soaking in 1% tetrazolium solution

Table 1. Growth of paprika (*C. annuum*) seedling after seed priming and water availability treatments

Combination treatment (KCL concentration and water availability)		Plant height (cm)	Leaf area (cm^2)	Fresh weight (gram)	Root to shoot ratio
Control (without priming)	50 FC	5.0^{ab}	2.39^{ab}	0.53^{ab}	1.00^{a}
	75 FC	8.6^{bcd}	3.25^{bc}	0.52^{ab}	2.32^{abc}
	100 FC	4.0^{a}	1.62^{a}	0.46^{a}	0.93^{a}
KCl 10 ppm	50 FC	5.3^{ab}	2.69^{ab}	0.38^{a}	1.37^{ab}
	75 FC	6.6^{abc}	2.21^{ab}	0.74^{ab}	1.78^{ab}
	100 FC	6.5^{abc}	2.30^{ab}	0.56^{ab}	1.75^{ab}
KCl 20 ppm	50 FC	6.5^{ab}	2.52^{ab}	0.37^{a}	1.64^{ab}
	75 FC	9.7^{cd}	2.67^{ab}	0.74^{ab}	1.29^{ab}
	100 FC	11.5^{de}	3.26^{bc}	1.11^{b}	2.23^{abc}
KCl 40 ppm	50 FC	11.1^{de}	3.40^{bc}	0.48^{a}	2.57^{ab}
	75 FC	14.9^{e}	3.28^{bc}	2.43^{c}	3.42^{c}
	100 FC	8.7^{bcd}	4.00^{c}	0.32^{a}	1.88^{ab}

Note: Different letter notation in the same column shows significantly different using Duncan's Multiple Range Test (DMRT) at a 95% confidence level; FC means field capacity

The plant heights from seeds that were not treated (control) and those that were treated with 10 ppm KCl were not significantly different in all variations of water availability, but treatment using KCl 20 and 40 ppm significantly increased plant height at all levels of water availability. The highest plant height resulted from 40 ppm KCl treatment.

At 50% field capacity water availability, the 40 ppm KCl treatment significantly increased paprika plant height. This means that seed priming treatment using KCl can stimulate paprika plants to grow well at low water availability. According to Putra et al. (2017), 50% level of field capacity similar than medium drought stress, so that seed priming using KCl has the potential to be used to increase the resistance of paprika plants to drought. Figure 2 shows the morphology of paprika seedlings that have been treated with different seed priming and water availability.

Besides the plant height parameters, according to Table 1, the combination of seed priming treatment using KCl and water availability also had a significant effect on the leaf area, fresh weight, and shoot:root ratio of paprika seedlings. The lowest leaf area resulted from control plants growing at 100% water availability; while the highest leaf area was produced from plants treated with 40 ppm seed priming and grew at 100% water availability. These results indicate that seed priming treatment could increase the growth of paprika plants. Higher leaf area can increase the area of light absorption for photosynthesis. The increased rate of photosynthesis causes increased production as well. In the 40 ppm seed priming treatment, the leaf area of paprika plants growing at various levels of water availability was not significantly different. These results indicate that seed priming treatment causes plants to grow well in conditions of low water availability (medium drought stress).

Plants express a dynamic response to sustain under stress conditions through morphological, physiological, and biochemical changes. The response of plants to drought stress conditions has been categorized into drought escape, drought avoidance, and drought tolerance (Marthandan et al. 2020). Determination of root to shoot is one of the adaptations of plants to drought stress. The root to shoot ratio depends upon the partitioning of photosynthate which may be influenced by environmental stimuli. The results of the ANOVA showed that seed priming treatment and water availability had a significant effect on the root to shoot ratio of paprika plants. The highest root to shoot ratio value was obtained from the seed priming treatment of 40 ppm, which grew at 75% water availability of field capacity. This indicates that under these conditions, the shoot biomass is low while the root biomass is high. In drought conditions, plants will increase their root growth in order to optimally absorb water and nutrients from the soil. Changes in the metrics of root-to-shoot relationships can compensate for moisture deficiency and maintain stomatal conductance under drought stress conditions (Kim et al. 2020). Drought stress can cause inhibition of cell division, enlargement and elongation caused by a lack of water availability (Rosawanti 2015). Drought stress can also cause a decrease in plant water potential, thereby reducing cell turgor. Then due to the decrease in cell turgor, it will inhibit the process of cell division and enlargement, so that the plant becomes short (Osakabe et al. 2014).

Biochemical parameters

Drought stress can alter the physiological and biochemical processes in plants. According to Mibei et al. (2017) the alteration processes including chlorophyll and carotenoid content, which is related to photosynthesis. The concentration of carotenoids and chlorophylls provides information about the level of stress experienced by the plant as well as its ability to endure these stresses. Therefore, due to the significant decrease in carotenoids during drought stress, it is evident that drought may lead to reduction in plant productivity. This is mainly by inhibiting growth and photosynthesis, and is one major limiting factor in agriculture worldwide leading to huge reductions in crop yield.

Table 2. Chlorophyll, carotenoid, and proline concentration of paprika (*C. annuum*) seedling after seed priming and water availability treatments

Combination treatment (KCL concentration and water availability)		Chlorophyll (mg/g of fresh weight)	Carotenoid (mg/g of fresh weight)	Proline (mg/g of fresh weight)
Control (without priming)	50 FC	11.84^{ab}	0.65^{a}	3.00^{cd}
	75 FC	13.4^{abc}	0.73^{ab}	1.26^{a}
	100 FC	15.48^{c}	0.80^{ab}	1.11^{a}
10 ppm	50 FC	13.44^{abc}	0.71^{ab}	2.60^{b}
	75 FC	13.22^{abc}	0.69^{ab}	2.85^{bc}
	100 FC	12.36^{ab}	0.87^{b}	3.00^{cd}
20 ppm	50 FC	12.80^{ab}	0.69^{ab}	3.48^{ef}
	75 FC	14.00^{bc}	0.74^{ab}	3.23^{de}
	100 FC	12.61^{ab}	0.62^{a}	3.58^{ef}
40 ppm	50 FC	11.54^{a}	0.68a	3.51^{ef}
	75 FC	11.50^{a}	0.67a	3.50^{ef}
	100 FC	13.47^{abc}	0.65a	3.73^{f}

Note: different letter notation in the same column shows significantly different using Duncan's Multiple Range Test (DMRT) at a 95% confidence level; FC means field capacity

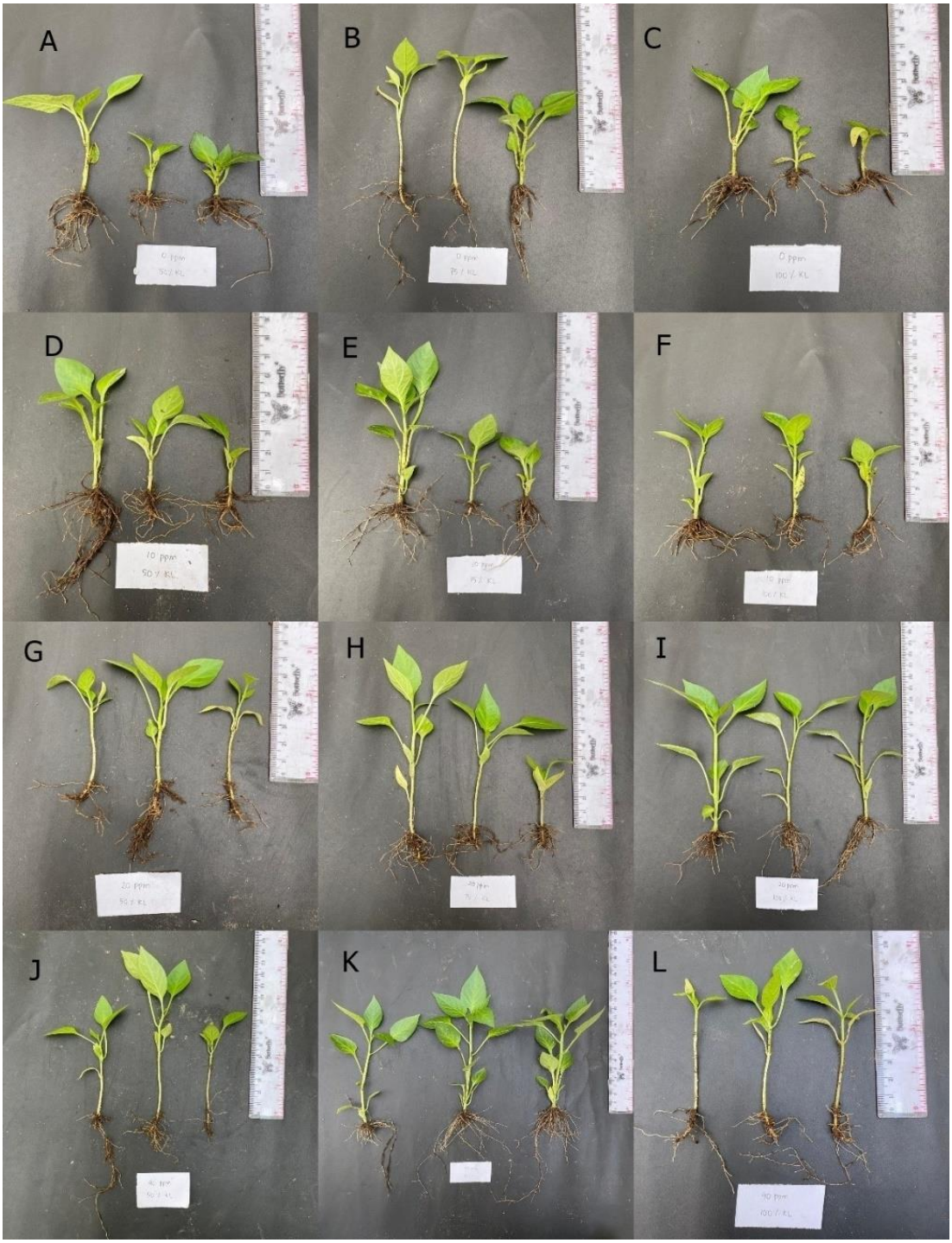


Figure 2. Seedling of paprika (*C. annuum*) two months after seed priming treatment using KCl and grown at different water availability. A: Control + 50%FC; B:control +75%FC; C:control+100%FC; D: 10ppm+50%FC; E:10ppm+75%FC; D: 10ppm+100%FC; G: 20ppm+50%FC; H:20ppm+75%FC; I: 20ppm+100%FC; J: 40ppm+50%FC; K: 40ppm+75%; L: 40ppm+100%FC

In this study, seed priming treatment significantly affected the chlorophyll content of paprika. The highest chlorophyll content resulted from the treatment of 20 ppm KCl at 75% field capacity. Seed priming treatment also significantly affected the carotenoid content of paprika, which is the highest carotenoid content resulting from the treatment of 10 ppm KCl at 100% field capacity. There was no pattern of increasing or decreasing levels of chlorophyll and carotenoids on water availability. According to Rustioni and Bianchi (2021), each plant has a different strategy in dealing with drought stress, for example, drought increases chlorophyll content in stems of *Vitis* interspecific hybrids. The concentration of photosynthetic pigments in woody tissues appeared to be strongly determined by genotypes.

Other metabolic alterations include accumulation of proline induced by drought stress in plants. Proline content of seedlings response to drought stress changed significantly compared to control (Table 1). Proline plays a vital role in maintaining optimal growth in plants under biotic stresses (Kurniawati et al. 2014; Nguyen et al. 2020). The significantly enhanced proline content in paprika leaf in drought stress is a response characteristic of plants like as osmotic adjustor under abiotic stresses. Proline promotes higher resistance in plant cells under adverse environmental conditions. In this study, seed priming treatment significantly affected the proline content of paprika. The highest proline content resulted from the treatment of 40 ppm KCl.

In conclusion, seed priming treatment using KCL at various concentration (10, 20, and 40 ppm) significantly affected the growth of paprika (*C. annuum*) that grow on variations in water availability. Seed priming treatment using KCl can increase the resistance of paprika plants to moderate drought conditions. Paprika resistance to drought is characterized by accumulation of proline and regulation of root to shoot ratio.

REFERENCES

Armita D, Arumyngtyas EL, Mastuti R. 2017. Tolerance level of three genotypes of cayenne pepper (*Capsicum frutescens* L.) toward drought stress of vegetative phase based on morphological and physiological responses. Intl J Chem Tech Res 10 (2): 183-192.

Bates IS, Waldren RP, Tare ID. 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39: 205-207. DOI: 10.1007/BF00018060.

Begna T. 2020. Effects of drought stress on crop production and productivity. Intl J Res Stud Agric Sci 6: 34-43. DOI: DOI: 10.20431/2454-6224.0609005.

de Rezende EM, Oliveira JA, Carvalho ER, Clemente ACS, Oliveira GE. 2017. Physiological quality of tomato seeds treated with polymers in combination with paclobutrazol. J Seed Sci 39 (4): 338-343. DOI: 10.1590/2317-1545v39n4164432.

Eivazi A. 2012. Induction of drought tolerance with seed priming in wheat cultivars (*Triticum aestivum* L.). Acta Agriculturae Slovenica 99: 21-29. DOI: 10.2478/v10014-012-0003-6.

Gayathri N, Gopalakrishnan M, Sekar T. 2016. Phytochemical screening and antimicrobial activity of *Capsicum chinense* Jacq. Intl J Adv Pharm 5: 12-20. DOI: 10.7439/ijap.v5i1.3025.

Hendry GAF, Grime JP. 1993. Methods on Comparative Plant Ecology, A Laboratory Manual. Chapman and Hall, London. DOI: 10.1007/978-94-011-1494-3.

International Seed Testing Association (ISTA). 2016 International Rules for Seed Testing: Annexe to Chapter 7. Seed Health Testing Methods. International Seed Testing Association.

Kakanga CJR, Ai NS, Siahaan P. 2017. Rasio akar: Tajuk tanaman padi lokal sulawesi utara yang mengalami cekaman banjir dan kekeringan pada fase vegetatif. J Bioslogos 7 (1): 1-5. DOI: 10.35799/jbl.7.1.2017.16208. [Indonesian]

Kim Y, Chung YS, Lee E, Tripathi P, Heo S, Kim KH. 2020. Root response to drought stress in rice (*Oryza sativa* L.). Intl J Mol Sci 21 (4): 1513. DOI: 10.3390/ijms21041513.

Kurniawan M, Izzati M, Nurchayati Y. 2010. Chlorophyll, carotenoid and vitamin C in some species of aquatic plants. Bull Anat Physiol 18: 28-40. DOI: 10.14710/baf.v18i1.2614.

Kurniawati S, Khumaida N, Ardie SW, Hartati NS, Sudarmonowati E. 2014. Pola akumulasi prolin dan poliamin beberapa aksesi tanaman terung pada cekaman kekeringan. J Agronomi Indonesia 42 (2): 136-141. DOI: 10.24831/jai.v42i2.8432. [Indonesian]

Marthandan V, Geetha R, Kumutha K, Renganathan VG, Karthikeyan A, Ramalingam J. 2020. Seed priming: A feasible strategy to enhance drought tolerance in crop plants. Intl J Mol Sci 21 (21): 8258. DOI: 10.3390/ijms21218258.

Matiu M, Ankerst DP, Menzel A. 2017. Interactions between temperature and drought in global and regional crop yield variability during 1961-2014. PLoS One 12 (5): 1-23. DOI: 10.1371/journal.pone.0178339.

Mibei EK, Ambuko J, Giovannoni JJ, Onyango AN, Owino WO. 2016. Carotenoid profiling of the leaves of selected African eggplant accessions subjected to drought stress. Food Sci Nutr 5 (1): 113-122. DOI: 10.1002/fsn3.370.

Mirmazloum I, Kiss A, Erdelyi E, Ladanyi M, Nementh EZ, Radacsi P. 2020. The effect of osmopriming on seed germination and early seedling characteristics of *Carum carvi* L. Agriculture 10 (94): 1-11. DOI: 10.3390/agriculture10040094.

Nawiri SO, Oduor RO, Jalemba AM. 2017. Genetic engineering of sweet potatoes (*Ipomoea batatas*) using isopentenyl transferase gene for enhanced drought tolerance. Asian J Agric 1: 85-99. DOI: 10.13057/asianjagric/g010206.

Naz F, Gul H, Hamayun M, Sayyed A, Husna, Sherwani SK. 2014. Effect of NaCl stress on *Pisum sativum* germination and seedling growth with the influence of seed priming with potassium (KCl and KOH). Am-Eur J Agric Environ Sci 14 (11): 1304-1311. DOI: 10.5829/idosi.aejaes.2014.14.11.748.

Nguyen TTQ, Trinh LTHE, Pham HBV, Le TV, Phung TKH, Lee SH, Cheong JJ. 2020. Evaluation of proline, soluble sugar and ABA content in soybean *Glycine max* (L.) under drought stress memory. AIMS Bioeng 7 (3): 114-123. DOI: 10.3934/bioeng.2020011.

Osakabe Y, Osakabe K, Shinozaki K, Tran LSP. 2014. Response of plant to water stress. Front Plant Sci 13: 1-8. DOI: 10.3389/fpls.2014.00086.

Putra AFA, Hanafi ND, Hamdan, Tafsin M, Mirwandhono RE. 2017. Production of *Brachiaria decumbens* grass at various levels of drought treatments. J Peternakan Integratif 5: 1-5. DOI: 10.32734/jpi.v513.2134.

Rosawanti P. 2015. Respon tumbuhan kedelai (*Glycine max* (L.) Merr.) terhadap cekaman kekeringan. J Daun 2 (1): 35-44. DOI: 10.33084/daun.v2i1.171. [Indonesian]

Rustioni L, Bianchi D. 2021. Drought increases chlorophyll content in stems of *Vitis* interspecific hybrids. Theor Exp Plant Physiol 33: 6978. DOI: 10.1007/s40626-021-00195-0.

Sayyari M, Ghanbari F. 2012. Effects of super absorbent polymer A200 on the growth, yield and some physiological responses in sweet pepper (*Capsicum Annuum L.*) under various irrigation regimes. Intl J Agric Food Res 1 (1): 1-11. DOI:10.24102/ijafr.v1i1.123.

Yusniwati, Sudarsono, Aswidinnoor H, Hendrastuti S, Santoso D. 2008. Pengaruh cekaman kekeringan terhadap pertumbuhan, hasil dan kandungan prolin daun cabai. J Agrista 12 (1): 19-27. [Indonesian]

Zhu ZH, Sami A, Xu QQ, Wu LL, Zheng WY, Chen ZP, Jin Xz, Zhang H, Li W, Yu Y, Zhou KJ. 2021. Effect of seed priming treatment o the germination and development of two rapeseed (*Brassica napus* L.) varieties under the co-influence of low temperature and drought. PLos One 16 (9) :e0257236. DOI: 10.1371/journal.pone.0257236.

ASIAN J NAT PROD BIOCHEM
Volume 20, Number 1, June 2022
Pages: 6-9

E-ISSN: 2580-2550
DOI: 10.13057/biofar/f200102

Kidney function status of Wistar rat treated with ethanolic extract of *Phyllanthus amarus* leaves owing to diethylnitrosamine intoxication

OJOCHENEMI EJEH YAKUBU[1,♥], DAVID CHRISTOPHER BANDO[1,2], MOSES ZEPHANIAH[1], ISAAC JOHN UMARU[1], MICHAEL SUNDAY ABU[1]

[1]Department of Biochemistry, Faculty of Pure and Applied Sciences, Federal University Wukari. PMB 1020 Katsina-Ala Road, Wukari, Taraba State, Nigeria. Tel./fax. +23-48-030000000, ♥email: oj4real_2007@yahoo.co.uk

[2]Bioresources Development Centre, National Biotechnology Development Agency. Jalingo, Taraba State, Nigeria.

Manuscript received: 6 January 2022. Revision accepted: 25 March 2022.

Abstract. *Yakubu OE, Bando DC, Zephaniah M, Umaru IJ, Abu MS. 2021. Kidney function status of Wistar rat treated with ethanolic extract of* Phyllanthus amarus *leaves owing to diethylnitrosamine intoxication. Asian J Trop Biotechnol 19: 6-9.* The kidney plays a central role in detoxification and excretion of harmful metabolites and therefore is susceptible to toxicity by xenobiotics. This research investigated the possible modulatory effect of *Phyllanthus amarus* Schumach & Thonn on diethylnitrosamine (DEN) induced nephrotoxicity in rats. Wistar rats received a single dose intraperitoneal injection of DEN. Twenty (20) rats were randomly allocated into four groups of five (5) rats each, and toxicity was induced using a single dose of DEN at 200 mg/kg intraperitoneally. Treatment was carried out using P. amarus extract at 200 mg/kg for 14 days, while silymarin was used as a standard drug orally at 5mg/kg. Kidney function parameters (Urea, Creatinine, sodium (Na^+)), Potassium (K^+), and Chloride (Cl^-) were determined. After 2 weeks, DEN-induced rats showed renal injury evidenced by the significant increase in circulating kidney function markers. Results showed that concurrent supplementation of *P. amarus* significantly ($p<0.05$) modulated kidney function markers and prevented renal tissue damage induced by DEN. In conclusion, *P. amarus* prevents DEN-induced nephrotoxicity via attenuation of oxidative stress and alleviation of the antioxidant defense system.

Keywords: Diethylnitrosamine, kidney, nephrotoxicity, *Phyllanthus amarus*, xenobiotics

INTRODUCTION

The genus *Phyllanthus*, which belongs to the Euphorbiaceae family, is one of the largest genera of flowering plants, with about 800 species found throughout the tropical and subtropical regions of both hemispheres, exhibiting a relatively wider range of habits such as annual or biennial herbs, shrubs, and trees.

Phyllanthus amarus Schumach. & Thonn. (Family: Euphorbiaceae) is widely found in all tropical and subtropical regions of the planet (Edeoga et al. 2006). The *P. amarus* is one of the most important herbs discovered recently in Nigeria and Akwa Ibom State, in particular. It is known among Ibibios and Efik's as ''oyomokisoamankeedem,'' Yoruba as "eyinolobe," Hausa as "geeron tsutsaayee," and Igbo as "Iteknwonwanazu" and in English as "leaf flower" or "chamber bitter" (Jagtap et al. 2016).

In several countries, the aerial section of *P. amarus* is highly prized in traditional and indigenous medicine for its healing powers, according to Foo and Wong (1992). This herb has long been used to cure liver problems and kidney stones worldwide. 'Chanca Piedra' is a Spanish word that means "stone breaker or shatter stone." 'Chanca piedra' has been utilized in South America to treat gall bladder and kidney stones and gall bladder infections. Antifungal, antibacterial, and antiviral properties have been discovered in *P. amarus* (Mirunalini and Krishnaveni 2010). Plant extracts of *P. amarus* can be utilized as blood purifiers for light malaria fevers and anemia, according to Heyde (1990). In combination with other herbs, *P. amarus* aids in the release of phlegm (Oudhia and Tripthi 2002); when the plant's leaves are boiled, it acts as a diuretic and can be used to treat diabetes, diarrhea, hepatitis, menstrual abnormalities, and skin conditions (Oudhia and Tripthi 2002).

In Suriname, a decoction of *P. amarus* is consumed with other plants to relieve stomachache, and constipation can also be treated with this plant (Oudhia and Tripthi 2002). Antioxidant properties of *P. amarus* extract to aid in eliminating free radicals from the human body (Nwanjo et al., 2007; Yakubu et al., 2019; Yakubu et al., 2021). Alkaloids, flavonoids, hydrolyzable tannins, major lignans, and polyphenols are secondary metabolites found in *P. amarus*.

Phyllanthus amarus has an antiurolithic action on the excretory system and treats kidney/gallstones, other kidney-related issues, appendix inflammation, and prostate problems (Bjelakovic et al., 2012). It is used in treating dyspepsia, colic, diarrhea, constipation, and dysentery due to its efficacy in gastrointestinal illnesses. The herb has been used to treat leucorrhoea, menorrhagia, and breast abscess in women, and it can also work as a galactagogue. For the treatment of chronic dysentery, the young branches of the plant are given as an infusion. Fresh leaf paste can heal wounds and treat white spots on the skin and jaundice. The stem juice can also be utilized to treat wounds. Urinary

difficulties and liver edema are treated with the whole plant extract. Stomach pain is treated with the root extract. The plant's floral paste is administered externally as a snake bite remedy (Chandewar and Dhongade 2013).

Over the years, there has been rising concern over the side effect of synthetic drugs on humans to treat various illnesses and diseases. There is also a lack of information on the health benefit of some plants, such as *P. amarus.* This research work is aimed at investigating the effect of ethanol extract of *P. amarus* leaves owing to diethylnitrosamine (DEN) intoxication on kidneys.

MATERIALS AND METHODS

Collection, identification, and preparation of plant (sample)

Fresh and well-grown leaves of *P. amarus* were harvested at the University Environment, Federal University Wukari, Taraba State, Nigeria. The leaves were identified at the herbarium of Biological Science, Federal University Wukari. The plant leaves were thoroughly washed and air-dried for two days, pounded to a fine powder using mortar and pestle, then stored and labeled in a dried container.

Plant ethanolic extraction

The fine powder was soaked in an adequate volume of ethanol (95%) measured at 1,300 mL, and the sample was stirred and allowed to stand for 48 hours before filtration. After that, the suspension was first filtered using a clean white sieving mesh and then filter paper (Whatman No.1) (Yakubu et al. 2016). The filtrate was then subjected to dryness to evaporate the ethanolic content at 45^{o}C under reduced pressure on a rotary evaporator to obtain an oily gel-like extract which was weighed with the use of analytical weighing balance (AHUS); the extract was stored in an air-tight container, corked and preserved for use. The aliquots portion of the crude plant extract was weighed and used for phytochemical screening, and also, a portion was given to the Wistar rat of the labeled and selected group (Yakubu et al., 2016).

Animal procurement and treatment

Twenty (20) albino rats (Wistar rats) of both sexes weighing 150-350g were purchased from a commercial breeder in the Obudu area in Cross River State, Nigeria. The rats were acclimatized for 14 days after randomization under standard laboratory conditions at (25±2^{o}C) and relative humidity of 60±5% and 12 hours of light/dark cycle before and during the experimental period. The rats were maintained on commercial poultry feed (growers mash) and water *ad libitum.*

Animal grouping and administration

Twenty (20) albino Wistar rats weighing 150-350 g of both sexes were divided into 4 groups (n=5 rats) and kept in aluminum cages. A single dose (200 mg/kg) of DEN was used to induce nephrotoxicity, while 200 mg/kg and 5 mg/kg of the extract and silymarin were used as treatment drugs daily for 14 days (Yakubu et al. 2016). (i) Group 1 (Normal control) received distilled water and fed only. (ii) Group 2 (DEN control) 200 mg/kg/ip. (iii) Group 3 (DEN and extract) 200mkg/kg/po. (iv) Group 4 (DEN and silymarin) 5 mg/kg/po.

Collection of blood samples for analysis

At the end of the experimental period (two weeks), the rats were withdrawn from the cages in each group on day 14 and placed in a desiccator containing cotton wool soaked in light chloroform to anesthetize the rats (Yakubu et al., 2016) partially. Blood samples were collected from the heart via cardiac puncture using a sterile syringe and needle. The blood sample was divided into two fractions: One fraction was put into plain sample tubes while whole blood samples were put in Ethylene diamine tetraacetate (EDTA) treated sample bottles (Yakubu et al., 2016). The serum was collected from the clotted sample in the sample container by letting it stand for 2 hours at room temperature to clot before centrifugation at 3,000 rpm for 20 minutes using an MSE England benchtop centrifuge. Sera obtained from each sample were gently separated using Pasteur pipettes and dispensed into respective dry specimen bottles labeled accordingly. These were kept frozen in a refrigerator until when needed for various biochemical assays. The blood samples collected into the EDTA bottles were corked immediately, shaken gently to allow the blood to mix with the anticoagulant and prevent clotting and cell hemolysis. The hematological analyses were carried out as soon as the blood sample was collected.

Determination of serum urea concentration

This was assessed using the method described by Fawcett and Scout (1960).

Principle: Urease breaks down urea into ammonia and carbon dioxide. In an alkaline medium, ammonia reacts with hypochlorite and salicylate to form dicarboxy indophenol, a colored compound. The reaction is catalyzed by sodium nitroprusside. The intensity of color produced is measured spectrophotometrically at 578 nm.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{salicylate} \longrightarrow \text{dicarboxyindophenols (blue compound)}$$

Procedure: Reagent (1 mL) containing sodium nitroprusside and urease was added into three clean test tubes labeled as a test sample, standard and reagent blank containing 0.01 mL sample, 0.01 mL standard reagent, and 0.01 mL distilled water, respectively. The content in each test tube was mixed and incubated at room temperature (25-30^{o}C) for 10 minutes. The absorbance of the test sample and standard were read against the reagent blank at 578 nm.

Calculation: The serum urea concentration was calculated using the formula below:

$$\text{Urea Conc. (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentrationof Standard}$$

BUN concentration (mg/dL) = 0.467 x urea concentration (mg/dL).

Determination of serum creatinine concentration

The colorimetric method was used to determine serum creatinine concentration according to Bertels and Bohme (1973).

Principle: Creatinine in the serum reacts with alkaline picrate to form colored complexes. The rate of formation of colored complexes is directly proportional to creatinine concentration. This reaction rate (intensity of orange color produced) is measured colorimetrically at 510 nm and compared with the standard.

Creatinine + Picric acid ⟶ NaOH Creatinine picrate (510 nm)

(Yellow) (Alkaline medium) (Orange)

Procedure: A working reagent (1 mL) containing picric acid and sodium hydroxide was added into two clean test tubes labeled sample test and standard, containing 0.1 mL of the test sample and 0.1 mL of standard solution. The content in each test tube was mixed, and after 20 seconds, the absorbance of the standard (ST1) and test sample (TS1) was read at 510 nm. Exactly 80 seconds later, absorbance for (ST2) and (TS2) of the standard and sample were read at 510 nm against distilled water (blank).

Calculation: The Concentration of creatinine in serum (mg/dl) was calculated using the formula below:

$$\text{Creatinine Conc.}(\text{mg/dl}) = \frac{TS2 - TS1}{ST2 - ST1} \; x \; \text{Concentration of Standard}$$

Where: ST: Standard, TS: Test Sample

Estimation of serum sodium, potassium and chloride ions

A flame photometer Model 143, equipped with an automatic diluter Model 144 (ratio of the dilution of 200:1) (Instrumentation Laboratory, Inc., Lexington, Mass., U.S.A.) was used. The calibration of the flame photometer was performed with twice distilled water and a standard having a Na^+ concentration of 140 mequiv/L and a K^+ concentration of 5 mequiv/L (Instrumentation Laboratory, Inc., Lexington, Mass., U.S.A). The instrument's stability was checked with the standard solution after each sample measurement.

Statistical analysis

The results were analyzed by one-way ANOVA, using SPSS statistical package version 20. All data were expressed as mean ± SD, and the difference between groups was considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The results of kidney function parameters such as Sodium (Na^+), Chloride (Cl^-), Potassium(K^+), Urea, and Creatinine obtained are presented in SD ± Mean error in Tables 1 and 2. Results showed that the extract could significantly ($p<0.05$) mitigate the harmful effect of the toxicity caused by DEN in the extract-treated group (Group 3) compared with Group 2, which was DEN-induced but not treated.

Phyllanthus amarus is an important medicinal plant used in ayurvedic medicine to treat diseases for over 2,000 years (Adedapo et al. 2005). The plant is also used in traditional medicine for the treatment of diseases. Scientific investigation revealed the therapeutic value of this medicinal plant; it showed that the plant contains several chemical constituents isolated and characterized and was found to be active against some diseases. For example, phyllanthin is a chemical compound isolated from *P. amarus* and reported hepatoprotective activity. This activity is associated with its radical scavenging activity (Krithika et al., 2009). The *P. amarus* has been used for years in different parts of the world for liver problems. The aqueous extract of *P. amarus* has also been used by Brazilians as a traditional medicine to treat stone disease (Barros et al. 2003) and jaundice and hepatitis (Mirunalini and Krishnaveni 2010). Scientific investigation has proven that the plant positively affects the hepatitis B virus and kidney stones.

Table 2. Urea and creatinine of Wistar rats Intoxicated by DEN and treated with ethanolic extract of *Phyllanthus amarus* leaves

Groups	Treatment	Urea	Creatinine
1	Normal	10.44±5.01[a]	5.12±3.21[b]
2	Den control	19.22±6.09[b]	12.56±2.37[b]
3	DEN + extract	8.4±1.37[a]	6.68±1.98[a]
4	DEN + silymarin	11.0±1.21[a]	4.64±3.82[a]

Results represent the mean ± standard deviation of group results obtained (n=5). Mean in the same row, having different letters of the alphabet is statistically significant ($p<0.05$) compared with the normal control (group one). Legend: Urea and creatinine

Table 1. Some electrolytes levels in Wistar rats intoxicated by DEN and treated with ethanolic extract of *Phyllanthus amarus* leaves

Groups	Treatment	Na^+	K^+	CL^-
1	Normal	138.6±12.34[a]	19.5±21.69[a]	145.0±25.14[a]
2	Den control	295.2±11.47[c]	38.42±23.22[b]	219.4±83.58[b]
3	DEN + extract	151.0±7.28[b]	25.58±20.84[a]	159.4±41.04[a]
4	DEN + silymarin	184.0±3.93[c]	21.26±20.58[a]	161.4±81.65[a]

Note: Results represent mean ± standard deviation of group results obtained (n=5). Mean in the same row, having a different letter of the alphabet is statistically significant ($p < 0.05$) compared with the normal control (group one). Legend: Sodium (Na^+), Potassium (K^+), Chloride (Cl^-)

The kidney helps maintain the body's homeostasis by reabsorbing important material and excreting waste products. Creatinine is a breakdown waste product formed in the muscle by creatinine phosphate metabolism. Creatinine is synthesized in the liver, passes into the circulation, and takes up almost entirely by skeletal muscle for energy production. Creatinine retention in the blood is evidence of kidney impairment.

Urea is the main end product of protein catabolism. Amino acid deamination takes place in the liver, which is also the site of the urea cycle, where ammonia is converted into urea and excreted through urine (David et al., 2014). It represents 90% of the total urinary nitrogen excretion. Urea varies directly with protein intake and inversely with the rate of excretion. Renal diseases that diminish urea's glomerular filtration rate will lead to its retention in the blood (David et al., 2012).

The kidney also maintains a marginal concentration of electrolytes in the body. Electrolytes are small inorganic ions prevalent in body fluid that are important in normal physiological functions (Palmer 2014). They are mainly sodium ion Na^+, chloride ion Cl^-, potassium ion K^-, bicarbonate ion HCO_3, and hydrogen ion H^+. The volume of extracellular fluid (ECF) depends on the body's sodium content because Na^+ and its salt are the major osmotic solute in ECF (Vasudevan et al., 2011). Renal regulation of these ions is controlled by renal sympathetic, atrial natriuretic peptide, and aldosterone actions which may result in reabsorption or excretion of these ions at the distal tubule of the nephrons. However, nephrology defects caused by xenobiotics such as CCl_4 and DEN toxicity may truncate these functions and result in irregular distribution of these ions in the ECF (Showkat et al. 2011).

In this research, upon induction of the rats with a toxic xenobiotic agent, DEN, the levels of the various kidney function parameters determined significantly ($p<0.05$) increased. However, the results of kidney parameters obtained showed that concurrent supplementation of *P. amarus* significantly ($p<0.05$) modulated kidney function markers and prevented renal tissue damage induced by DEN. The *P. amarus* may prevent DEN nephrotoxicity via attenuation of oxidative stress and activation of the body's antioxidant defense mechanism, which agrees with Showkat et al. (2011), where aqueous rhizome extract extracts of *Podophyllum hexandrum* Royle was able to reverse kidney and lung functions owing to its antioxidant propensity.

In conclusion, kidney function parameters (urea, creatinine, sodium, potassium, chloride) were significantly ($p<0.05$) reversed after extract treatment which was an indication of amelioration of the harmful effects of the toxicity caused by DEN by the *P. amarus* leaves extract.

REFERENCES

Adedapo AA, Adegbayibi AY, Emikpe BO. 2005. Some clinico-pathological changes associated with the aqueous extract of the leaves of *Phyllanthus amarus* in rats. Phytother Res 19: 971-976. DOI: 10.1002/ptr.1768.

Barros ME, Schor N, Boim MA. 2003. Effects of an aqueous extract from *Phyllanthus niruri* on calcium oxalate crystallization in vitro. Urol Res 30: 374-379. DOI: 10.1007/s00240-002-0285-y.

Bertels H, Bohmer M. 1973. Serum creatinine determination without protein precipitation. Clin Chim Acta 37: 193. DOI: 10.1016/0009-8981(72)90432-9.

Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. 2012. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Cochrane Database Syst Rev 3 (3): 71-76. DOI: 10.1002/14651858.CD007176.pub2.

Chandewar AV, Dhongade H. 2013. Pharmacognostical and Phytochemical studies of *Phyllanthus amarus* leaves. Intl J Biomed Adv Res 4: 383. DOI: 10.7439/ijbar.v4i6.405.

David MS, Jeff SC, Nigel B, David WJ, Glenda CG. 2012. Oxidative stress, antioxidant therapies and chronic kidney disease. Nephrology 17: 311-321. DOI: 10.1111/j.1440-1797.2012.01572.x.

David WI, William EM, Jeff MS. 2014. Urea and ammonia metabolism and the control renal nitrogen excretion. Clin J Am Soc Nephrol 10 (8): 1444-1458. DOI: 10.2215/CJN.10311013.

Edeoga HO, Omosun G, Awomukwu DA. 2006. Tannins and calcium oxalate crystals in lamina of some *Phyllanthus* species. Intl J Mol Med Adv Sci 2: 326-329.

Fawcett JK, Scout JE. 1960. A rapid and precise method for the determination of urea. J Clin Pathol 13: 156. DOI: 10.1136/jcp.13.2.156.

Foo LY Wong H. 1992. Phyllanthusiin D, an unusual hydrolysable tannin from *Phyllanthus amarus*. Phytochemistry 31 (2): 711-713. 10.1016/0031-9422(92)90071-W.

Heyde H. 1990. Medicijnplanten in Suriname. (Den dresiwiwiri foe Sranan). Medicinal Plants in Suriname. Uitg. Stichting Gezondheidsplanten Informaite (SGI), Paramaribo, Suriname.

Jagtap S, Khare P, Mangal P, Kondepudi KK, Bishnoi M, Bhutani KK. 2016. Protective effects of phyllanthin, a lignan from *Phyllanthus amarus*, against progression of high fat diet induced metabolic disturbances in mice. RSC Advances 6 (63): 58343-53. DOI: 10.1039/C6RA10774E.

Krithika R, Mohankumar R, Verma RJ, Shrivastav PS, Mohamad IL, Gunasekaran P, Narasimhan S. 2009. Isolation, characterization and antioxidative effect of Phyllanthin against CCl_4-induced toxicity in HepG2 cell line. Chem-Biol Interact 181: 351-358. DOI: 10.1016/j.cbi.2009.06.014.

Mirunalini S, Krishnaveni M. 2010. Therapeutic potential of *Phyllanthus emblica* (amla): The ayurvedic wonder. J Basic Clin Physiol Pharmacol 21 (1): 93-105. DOI: 10.1515/JBCPP.2010.21.1.93.

Nwanjo HU, Oze G, Okafor MC, Nwosu D, Nwankpa P. 2007. Protective role of *Phyllantus niruri* extract on serum lipid profiles and oxidative stress in hepatocytes of diabetic rats. Afr J Biotechnol 6 (15): 1744-1749. DOI: 10.5897/AJB2007.000-2256.

Oudhia P, Tripthi RS. 2002. Prospect of cultivation of medicinal plants in Chhattisgarh, India. In: Crop Improvement, Production Technology, Trade and Commerce 211-235. Sci Tech Publishing LLC, Houston, USA

Palmer BF. 2014. Regulation of potassium homeostasis. Clin J Am Soc Nephrol 10 (6): 1050-1060. DOI: 10.2215/CJN.08580813.

Showkat AG. Ehtishamul H, Abid H, Yasrib Q, Zahi M, Bilal, AZ, Akbar M, Mahammad AZ. 2011. Carbon tetrachloride induced kidney and lung tissues damage and oxidative activities of aqueous rhizome extract of *Podophyllum hexandrum*. BMC Complement Altern Med 11 (17): 1472-6882. DOI: 10.1186/1472-6882-11-17.

Vasudevan DM, Sreekumar S, Kanan V. 2011. Biochemistry for medical students. Jaypee Brothers Medical Publishesr Limited. 6th Edition. New Delhi, India.

Yakubu OE, Abu MS, Akighir J, Onuche JI, Arabi A. 2021. Comparative determination of total antioxidant effects of ethanol extract of *Phyllanthus amarus* leaves. Asian J Nat Prod Biochem 19: 81-85. DOI: 10.13057/biofar/f190206.

Yakubu OE, Nwodo OFC, Shaibu C, Tatah SV, Abah MA, Sunday G. 2019. In vitro determination of antioxidant activities of the fractions obtained from *Adansonia digitata* L. (baobab) stem bark ethanolic extract using different parameters. Curr Trends Biomed Eng Biosci 17 (5): 555973. DOI: 10.19080/CTBEB.2019.17.555973.

Yakubu OE, Obot AC, Dawoye Y. 2016. Effect of aqueous extract of *Hymenocardia acida* leaves on Aluminium chloride-induced toxicity in male albino rats. J Anal Pharm Res 3 (2): 00049 DOI: 10.15406/japlr.2016.03.00049.

ASIAN J TROP BIOTECHNOL
Volume 19, Number 1, June 2022
Pages: 11-19

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c190103

Hydrolases secreting, heavy metal resistant halophilic bacteria isolated from metal dumpsites

CHIOMA FLORENCE MGBODILE[1], UZOAMAKA OTUTU[1], STEPHANIE ONUOHA[1], UCHENNA EZE[1], TOBECHUKWU UGWUOJI[2], OBIANUJU NNABUIFE[1], TOCHUKWU NWAMAKA T. NWAGU[1,♥]

[1]Department of Microbiology, Faculty of Biological Sciences, University of Nigeria. Nsukka, Enugu State, Nigeria. Tel.: +2349033668791, ♥email: tochukwu.nwagu@unn.edu.ng

[2]Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University. PMB 5025, Awka, Anambra State, Nigeria

Manuscript received: 17 January 2022. Revision accepted: 9 June 2022.

Abstract. *Mgbodile CF, Otutu U, Onuoha S, Eze U, Ugwuoji T, Nnabuife O, Nwagu TNT. 2022. Hydrolases secreting, heavy metal-resistant halophilic bacteria isolated from metal dumpsites. Asian J Trop Biotechnol 19: 11-19.* Microorganisms with the potential to accumulate heavy metals are currently under investigation for application in environmental detoxification. The current study aimed to isolate heavy metal tolerant bacteria from metal dump sites. The soil samples were sourced from two metal dumpsites in Eastern Nigeria. The pH and metal content of the samples were evaluated. Microorganisms were screened for tolerance of lead, copper, silver and chromium. Isolated strains were characterized and identified by molecular techniques. All isolates grew in 1000 ppm Pb, Cu and Cr. The optimal temperature for their growth was 37°C. While isolates B and C grew optimally at 12.5% NaCl, the growth rate increased for isolate A (i.e., 7.5%>12.5%>15%). The three isolates produced extracellular protease, and inulinase isolates B and C produced amylase, while isolating A produced xylanase. Isolate A, B, and C were identified as *Pseudomonas asiatica*, *Sphingobacterium caeni*, and *Burkholderia cenocepacia*. The bacteria were resistant to a wide range of antibiotics, including ampicillin (30 mcg/disc), ceporex (10 mcg/disc), and streptomycin (30 mcg/disc). These results indicate that the bacterial strains have the potential as sources of inoculants for the bioremediation of heavy metal contaminated environments and application in various industrial processes where metal-resistant organisms and their hydrolases are required.

Keywords: *Burkholderia* sp., environmental remediation, heavy metal tolerance, *Pseudomonas* sp., *Sphingobacterium* sp.

INTRODUCTION

Heavy metal contamination is a major environmental problem due to its toxicity (Juhaeti et al. 2004; Gurave et al. 2015; Irawati et al. 2021). Urbanization and industrialization have resulted in several activities that interact with or utilize various heavy metals in one form or another; a typical example is mining (Gautam et al. 2016; Irawati et al. 2017). As a result, high concentrations of heavy metals, including silver, copper, cadmium, zinc, iron, manganese, nickel, and lead, pollute the environment. The current widespread contamination of the air, surface waters, ground waters, sediments, and soil with heavy metals constitute a serious threat to all living organisms; since most metals are highly toxic and not easily degraded, unlike organic molecules, they indefinitely persist in the environment (Zulkali et al. 2006). Cadmium is a non-essential metal, not involved in any known biological process. However, it is fast accumulating in our environment due to its wide application in many industrial processes, including the pulp and paper industry, the production of copper alloys, electroplating, alkaline batteries, fertilizer, metal mining, and refining. Unfortunately, low cadmium concentrations (about 0.001-0.1 mg/L) are harmful to organisms, and accumulation causes much damage to plants leading to poor productivity (Chellaiah 2018). The environmental stress caused by these heavy metals generally decreases soil bacterial diversity and activity, leading to a reduction in the total microbial biomass, reduced numbers of specific populations, and a shift in the microbial community (Zulkali et al. 2006)

Cleaning metal-contaminated sites are required to avoid environmental degradation and preserve human health. Standard methods for environmental clean-up involve physicochemical methods, including electrodialysis, ion exchange, ultrafiltration, and precipitation (Hashim et al. 2011). Owing to the high cost, low efficacy, and environmentally unfriendly nature of most of these methods, alternative techniques are currently being researched and adopted (Wang and Chen et al. 2009). The application of bacterial biomass in environmental clean-up has received great attention in recent years, especially due to its cost-effectiveness and eco-friendly nature (Wang and Chen 2009). The response of the soil microbial population to heavy metal contamination provides a relevant model for environmental studies. Different microorganisms display various reactions to toxic metals that confer them with a range of metal tolerance (Chihomvu et al. 2014). Many studies have shown that these heavy metals affect the microbial population differently, including morphology, growth, density, and biochemical activities (Kathiravan et al. 2011; Abd et al. 2013). Microbial response to toxic metals depends on the microbial strain and the type of metal implicated (Mohamed and Abor-Amer 2012). Environments heavily contaminated with heavy metals harbor organisms that can deal with pollution, including

prokaryotes and eukaryotes. The metal resistance and ability to convert the toxic metals to less harmful states make them potentially useful in bioremediation and other industrial applications (Gurave et al. 2015).

This study aimed to isolate and characterize bacterial isolates from soil from heavy metal dump sites and evaluate their potential for heavy metal resistance, sugar and salt tolerance, and enzymatic activities.

MATERIALS AND METHODS

Sample collection

Soil samples from the surface layer (0-20 cm) were collected from two metal dumpsites in Enugu and Anambra state, both in the South-Eastern part of Nigeria. The soil samples were placed into labeled plastic polyethene bags, quickly transported to the laboratory for microbiological analysis and stored at 4ºC till further use. A small portion of the sample was pulverized with a pestle in a sterile mortar, air-dried and sieved with a 2-mm metal screen, and then kept aside for physical and chemical analysis (Jiang et al. 2017).

Determination of soil pH

According to Aka and Babalola (2017), the soil samples were analyzed for pH. First, the soil sample (5 g) was suspended in 12.5 mL of distilled water and allowed to stand for 30 min. Then, pH was recorded using a calibrated glass electrode pH meter (Apera Instruments, LLC).

Total metal (Pb, Cd, and Cu) concentration in soil

To determine the metals present in the soil samples, the samples were first digested with a microwave digester per the standard method by Aka and Babalola (2017). Afterward, the samples' volume was adjusted to 50 ml each with distilled water, and the concentrations of Pb, Cd, and Cu in the sample were measured using the Atomic Absorption Spectrophotometer, AA-7000 (Shimadzu Scientific Instruments).

Isolation and screening for heavy metal-resistant bacteria

Soil sample (1 g) was suspended in an enrichment medium comprising 10 mL of sterilized peptone water containing 500 ppm metal salt (chloride of Cd, Pb, Hg, or Cu) and agitated continuously at 24+2ºC for 18 h (Singh et al. 2010). After 18 h, 0.1 mL of each incubated sample was placed on Luria-Bertani (LB) agar plates by the spread plate method. The plates were incubated at 24+2ºC for 2-3 days and observed for visible colonies.

In order to isolate strains tolerant to high concentrations of the heavy metals, distinct colonies were subcultured (streak method) onto LB agar plates containing increasing concentrations of the heavy metal salts (500-1000 ppm). Isolates displaying the greatest resistance to the metals were selected for further studies.

Determination of the cellular and biochemical properties of the isolates

The colonies grown on LB plates were analyzed morphologically and then Gram-stained to observe under a light microscope using oil immersion at 100 x to 400x. Biochemical tests were carried out, including sugar utilization, urease and indole production, nitrate reduction, catalase and oxidase detection tests (Khosro et al. 2013).

Temperature tolerance of the heavy metal-resistant bacterial strains

The isolates (20 µL of 12-hour culture) were inoculated into 20 mL of sterilized LB broth containing 500 ppm of Pb, Cd, or Cu chloride and incubated at different temperatures (10ºC, 25ºC, 37ºC, and 40ºC) for 48 h to determine the effect of temperature on the bacterial growth. After incubation, each sample's optical density (OD) was measured at 600 nm using a UV spectrophotometer.

Glucose tolerance of the heavy metal-resistant bacterial strains

The glucose tolerance of the bacterial isolates was determined according to Aka and Babalola (2017). LB broth (20 mL) containing increasing concentrations of glucose (8%, 12%, 16%, and 24%) were inoculated with 100 µL (OD = 0.5) of 12-hour bacterial cultures and incubated at 37ºC for 24 h. The optical density was determined at 600 nm using a UV spectrophotometer.

Screening for the ability to produce selected enzymes

The microbial isolates were screened to produce hydrolytic enzymes by using a modification of the method by Veras et al. (2018). Agar plates containing casein, starch, cellulose, xylan, Tween 80 and inulin were used to screen for protease, amylase, cellulase, xylanase, lipase and inulinase, respectively. First, a loopful of the organism was streaked on the agar plate and incubated for 48-72 h. Afterward, the plates were flooded with 0.2 g/L potassium iodide for 5 min and observed for the presence of clear zones around bacteria colonies.

Determination of antibiotic resistance

According to Kirby Bauer's methods, the antibiotic resistance of our isolates was determined by a susceptibility test (Bauer et al. 1966). The isolates were enriched in nutrient broth for 24 h and swabbed aseptically onto freshly prepared Muller Hinton agar plates (Neethu et al. 2015). Standard antibiotic-impregnated discs were then placed on the plates and incubated for 24h at 37ºC. The diameter of the inhibition zone was measured, and results were recorded in terms of Resistant (R), Susceptible (S), or Intermediate (I), using the standard antibiotic disc chart.

Molecular identification and phylogenetic analysis

The DNA extraction of the bacterial isolates was performed using the ZR Bacterial DNA Miniprep Kit. The polymerase chain reaction (PCR) was performed on the extracted DNA samples using universal bacterial primers 27F (5′ AGA GTT TGA TCM TGGCTC AG 3′) and 1525R (5′ AAG GAG GTG WTC CAR CCG CA 3′)

(Chouari et al. 2005). The PCR cycle started with an initial denaturation step at 94ºC for 5 min, followed by 36 cycles of denaturation at 94ºC for 30 sec. Annealing was at 55ºC for 30 sec and elongation at 72ºC for 45 sec, followed by a final elongation step at 72ºC for 7 min, and cooling to 4ºC. DNA amplification was evaluated by electrophoresis of 10 µL of each PCR product in 1% (w/v) agarose gel as described by Abor-Amer et al. (2014). The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems, while the sequencing kit used was the Big Dye terminator v3.1 cycle sequencing kit. The genetic analysis was conducted using the Bio-Edit and MEGA 6 software. Bioinformatics analysis of the sequencing results was performed using the blastn algorithm (Altschul et al. 1997), at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis was done using the neighbor-joining method in the MEGA-X software (Kumar et al. 2018). The Neighbor-Joining method was applied to determine the evolutionary history and the Kimura 2-parameter method for the distances (Kimura 1980).

RESULTS AND DISCUSSION

Determination of soil pH and total Pb, Cd, and Cu concentration

The soil pH of samples was recorded as 8.2 and 6.7, collected from Abagana and Enugu, respectively. The Atomic Absorption Spectrophotometric (AAS) analysis of the soil samples revealed that the order of concentration of Cu, Pb, and Cd was 6.42, 3.00, and 0.12 ppm, respectively, for the Abagana soil sample, and 0.50, 0.39 and 0.09 ppm, respectively for Enugu soil sample (Figure 1). It was observed that the Abagana soil sample had relatively higher levels of heavy metals than the Enugu soil sample. While Cu was the highest contaminating metal (among those evaluated) in the Abagana soil sample, Pb was the highest contaminating metal in the Enugu sample. Heavy metal contamination in soils is one of the world's major environmental problems, posing significant risks to the ecosystems and the general public (Sheng et al. 2008). Several environments around us are battling heavy contamination, and environmental pollution is fast spreading worldwide as industrialization increases (Gautam et al. 2016). Copper, chromium, cadmium, and lead are the most commonly implicated in these heavy metals (Mendez et al. 2008). Though traces of heavy metals serve as essential co-factors to some enzymatic reactions, at high concentrations, these metals may become toxic to living organisms due to the inhibition of metabolic reactions (Hussein et al. 2004).

Screening for heavy metal tolerant microorganisms

Three isolates were obtained based on the ability to grow on 1000 pm of salts containing Cu^{2+}, Pb^{2+}, and Cd^{2+}. One isolate (Isolate A) was obtained from the Abagana soil sample, while two isolates (Isolate B and Isolate C) were from the Enugu soil samples. The pH of the soil samples varied from slightly acidic to slightly alkaline, depending on the source. The soil pH is an important parameter that strongly affects the behavior of metal ions present in a terrestrial environment. It also affects the soil solubility and ability to form chelates with other soil constituents (Aka and Babalola 2017). All the isolates could not grow in the presence of Hg^{2+} (result not shown). Heavy metal pollution of soil changes its microbial communities (Pacwa-Plociniczak et al. 2018). The metal tolerance of the isolated bacteria was in the order of Cd>Pb> Cu>Hg, Pb> Cd>Cu>Hg, and Pb>Cu>Cd> Hg for isolates A, B, and C, respectively. Table 1 shows the result of the isolates' growth rate under different concentrations (500, 600, and 1000 ppm) of the heavy metal salts (Cu, Pb, and Cd). Very good growth was recorded for isolates A when grown on 500 ppm of all the metal salts tested. Though very good growth was observed when isolate C was grown on 500 ppm Pb^{2+} and Cu^{2+}, the same was not the case on Cd^{2+}. Isolates A, B, and C had good growth on 1000 ppm Pb^{2+} but were inhibited by 1000 ppm Cu^{2+}. Interestingly, isolate A had very good growth on 1000 ppm Cd^{2+}, while isolates B and C had poor growth.

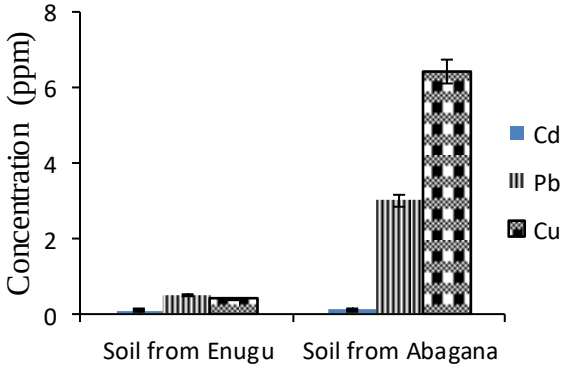


Figure 1. The concentration of heavy metals in the soil sample

Table 1. The growth rate of the heavy metal resistant bacteria in varying concentrations of the metal salts

Isolate	Conc. (ppm)								
	Pb^{2+}			Cu^{2+}			Cd^{2+}		
	500	600	1000	500	600	1000	500	600	1000
A	+++	++	++	+++	++	+	+++	+++	+++
B	++	++	++	+++	++	+	++	++	+
C	+++	+++	++	+++	++	+	++	++	+

Note: +++: very good, ++: good, + poor, - : no growth

When the isolates were subjected to increasing concentrations of heavy metal, it was discovered that the time required for colony formation was longer, suggesting that the organisms had to constantly adjust their cellular environments to accommodate the heavy metal stress. Microbial colonies' sizes also varied; for instance, in a lower concentration, such as 500 ppm, the colonies appeared robust and profuse, while in higher concentration (1000 ppm), the colony sizes were much smaller. The isolates thrived in 500 ppm of Pb^{2+}, which may be due to the high concentration of Pb^{2+} in the soil samples. High amounts of heavy metals in the soil confer resistance to the soil microbial population (Mohamed and Abor-Amer 2012). The Pb concentration for Anambra and Enugu soil was 3.0 ppm and 0.50 ppm, respectively. Though lead is not a micro-nutrient, higher concentrations (600 and 1000 ppm) did not significantly inhibit the growth of the bacterial strains, although the colony sizes were slightly reduced. A low concentration of Cu^{2+} (500 ppm) strongly promoted the bacterial strains' growth, which could be attributed to Cu^{2+} being a micro-nutrient required by living organisms in minute concentrations (Gurave et al. 2015). Another responsible factor may be the concentration of Cu^{2+} in the soil samples (a concentration as high as 6.40 ppm for Anambra soil and 0.39 for Enugu soil). It was, therefore, not surprising that the isolates could thrive in the copper-containing medium (Raja et al. 2009). However, with increasing Cu^{2+} concentrations, a decline in bacteria growth was observed; at 1000 ppm, growth was very poor. Though a micro-element for some metabolic activities, at high concentration, it could play inhibitory roles to organisms that previously promoted growth in minute concentrations (Abd et al. 2013). Although the soil samples did not contain higher cadmium concentrations than copper or lead, the isolates were relatively tolerant to the Cd^{2+} even at 1000 ppm. Pacwa-Plociniczak et al. (2018) isolated a Cd-resistant *Sphingomonas* sp. from a long-term heavy metal polluted soil; and concluded from their observations that Cd-resistant bacteria are common in long-term polluted soils. Of particular interest is *Pseudomonas asiatica*, which displayed a notable growth trend in cadmium supplemented media maintain a relatively same density of growth at all the concentrations investigated. Thus *P. asiatica* strain A could be said to be 'cadmium loving' loosely speaking. Ahirwar et al. (2016) isolated a Cd^{2+} and Pb^{2+} resistant *Pseudomonas spp*. among other isolates from an industrial soil in Central India. Similar to our work, Hg was the most toxic of the metals investigated. Ahirwar et al. (2016) attributed the high Pb^{2+}/Cd^{2+} resistance of isolated Pseudomonas strain to the high concentration of these metals in the soil from which these strains were isolated.

Characterization of the isolates

The physicochemical characteristics of the selected isolates are shown in Table 2. The isolates showed a similar form (circular), elevation (convex), and margin (entire) but different colors when growing on the agar containing the different metals. The isolates were Gram-negative, in line with findings that Gram-negative bacteria dominate metal-contaminated soil (Frostgard et al. 1993; Sulowicz et al. 2011; Pacwa-Plociniczak et al. 2018). They were catalase-positive and negative for the indole test and methyl red. Isolates A, B, and C were able to utilize glucose and sucrose but unable to utilize dulcitol. While isolate C showed weak growth in mannose and lactose, isolates A and B could not utilize these sugars.

The isolates' ability to secrete industrially relevant enzymes such as protease, amylase, cellulase, xylanase, and inulinase was evaluated. The enzyme assay result showed the bacterial strains varying abilities to secrete extracellular hydrolases when growing on a solid medium. While all the isolates could secrete extracellular proteases and inulinase, only B and C could synthesize amylase and cellulase. Isolates A and C were xylanase producers, while B was negative for xylanase production (Table 2).

The effect of temperature on the growth of the isolates on different is shown in Figure 2. All the isolates grew poorly at 10ºC irrespective of the metal present, except for isolate B grown in a medium containing Pb^{2+}. Optimum growth rates for all our isolates were generally recorded between 25-37ºC, regardless of the heavy metal present. A drastic drop in growth occurred when the temperature increased to 45ºC. There was no remarkable difference when isolate B incubated in a medium containing Pb^{2+} was grown over the temperatures of 10, 25, and 37ºC. There was a rapid decline in growth following a temperature increase to 45ºC. Growth characteristics, metabolism, and the microorganisms' cell membrane are altered due to temperature variations (Kathiravan et al. 2011). The result shows that the isolated bacterial strains were mesophilic, which aligns with the sample source's environmental temperature and climatic condition.

Considering the presence of varying solutes in the environment and their effects on microbial growth and metabolite production, we investigated the effect of low to a high concentrations of salt (NaCl) and glucose on the heavy metal-resistant strains. The isolates exhibited varying levels of NaCl tolerance. Isolate A grew with increasing concentration of NaCl (10% > 12.5% > 15.0%). Isolates B and C grew optimally in the presence of 12.5% NaCl concentration. A further increase in NaCl concentration to 15% led to a decrease in microbial growth (Figure 3). As seen in Figure 4, all the isolates grew at glucose concentrations in the range of 8-24 %; however, the highest growth at 8% glucose concentration was observed for isolates B and C. As expected, the varying concentration of these agents influenced the bacterial growth. Isolate A grew exponentially in the presence of 10-15% salt concentration, indicating its moderate or extreme halophile nature (Singh et al. 2019; Irwin 2020).

Molecular identification

The size of the fragments produced from the PCR amplification of the 16S rRNA gene of each bacterium was determined. The variable sized DNA fragments of the bacterial isolates can be seen in Figure 4. The PCR products were sequenced and matched to the available sequences in the NCBI database. Table 3 indicates that isolates A was *P. asiatica* strain A (NCBI Accession

number MW304002). Isolate B was identified as *Sphingobacterium caeni* strain B (NCBI accession number MW304003), while isolate C was identified as *Burkholderia cenocepacia* strain C (NCBI accession number MW304004). The evolutionary history of bacterial strains was inferred using the Neighbor-Joining method. Figure 5 shows the phylogenetic relatedness of *P. asiatica* strain A, *S. caeni* strain B and *B. cenocepacia* strain C to other closely related strains. It is established that the soil harbors varying species of *Pseudomonas* (Noura et al. 2009), and *Burkholderia* (Hall et al. 2015). *Sphingobacterium* species have been isolated from the soil and activated sludge (Sun et al. 2013; Feng et al. 2014; Cheng et al. 2019). There are available reports of moderately halophilic *Pseudomonas* species isolated from saline and hypersaline environments such as brined foods and water bodies (Korcan et al. 2015; Patel and Saraf 2015). However, *P. asiatica* is a novel species isolated from a stool sample from a hospitalized patient in Japan (Tohya et al. 2019). More recently, *P. asiatica* C1 capable of synthesizing coenzyme B12 while growing aerobically on glucose was isolated from activated sludge in Korea's waste treatment plant (Nguyen et al. 2021). This was the first report to isolate a heavy metal tolerant halophilic *P. asiatica* from the soil. Potential applications of halophiles include producing compatible solutes, biopolymers (polyhydroxyalkanoates and polysaccharides) and enzymes. Halophiles are also employed in biodegradation and remediation, enhanced oil recovery, cancer detection, and drug screening (Waditee-Sirisattha et al. 2016). Feng et al. (2014) reported that *Sphingobacterium paludis* sp. nov and *S. caeni* sp. nov. did not grow in NaCl concentrations above 5% (w/v). In comparison, the *S. caeni* strain A isolated in the current study grew in 15% salt concentration while the optimum concentration required for growth was 12.5% (w/v). Several *Burkholderia* species are halotolerant and help ameliorate plant salt stress (Arora et al. 2020). Recently reported cadmium, nickel, and mercury tolerant *B. cenocepacia* isolated from plant environments (Torres et al. 2019). *Burkholderia* species are mainly endophytes with heavy metal tolerant abilities used in plant growth promotion and environmental remediation (Jiang et al. 2008; Liu et al. 2019). Inoculation of soil with heavy metal-resistant bacteria enhances the expression of metal stress-related genes in plants with a consequent increase in plant growth and yield (Liu et al. 2018).

Table 2. Cultural, morphological and biochemical properties of the heavy metal-resistant bacterial isolates

Characteristics	Isolates		
	A	B	C
Morphology			
Form	Circular	Circular	Circular
Elevation	Convex	Convex	Convex
Margin	Entire	Entire	Entire
Colony colour when grown on			
Pb^{2+}	Brown	Silvery	Light
Cd^{2+}	Creamy	Whitish	Silvery white
Cu^{2+}	Whitish	Yellowish	Yellowish
Biochemical characteristics			
Gram stain	-	-	-
Catalase	+	+	+
Indole	-	-	-
Nitrate reduction	+	-	+
Methyl red	-	-	-
Citrate	+	+	-
Utilization of carbohydrate			
Glucose	+	+	+
Sucrose	+	+weak	+
Dulcitol	-	-	-
Mannose	-	-	+weak
Lactose	-	-	+weak
Production of some hydrolases			
Protease	+	+	+
Amylase	-	+	++
Cellulase	-	+	+
Xylanase	+	-	+
Inulinase	+	+	+

Note: ++ : very good; + : good; +weak : poor; - : negative

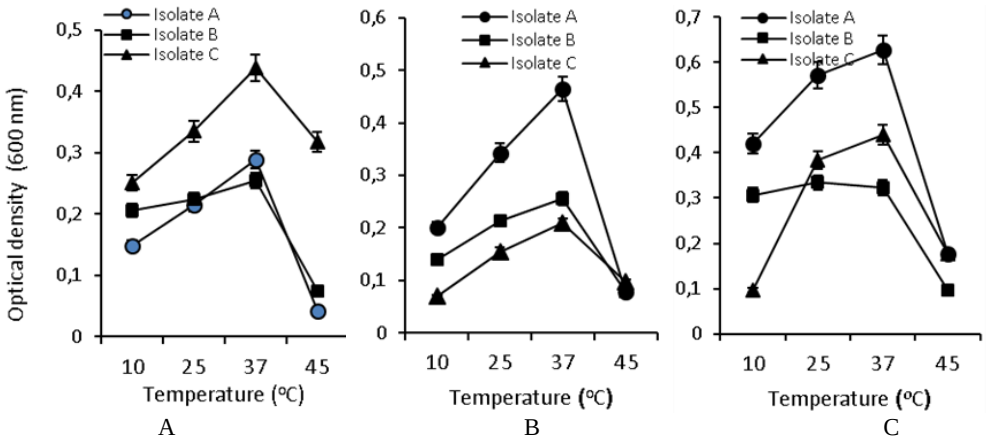


Figure 2. Growth of the isolate at varying temperatures in the presence of (A) Cu^{2+}, (B) Cd^{2+}, and (C) Pb^{2+} salts. Luria Bertani (LB) broth was supplemented with 500 ppm of the salt

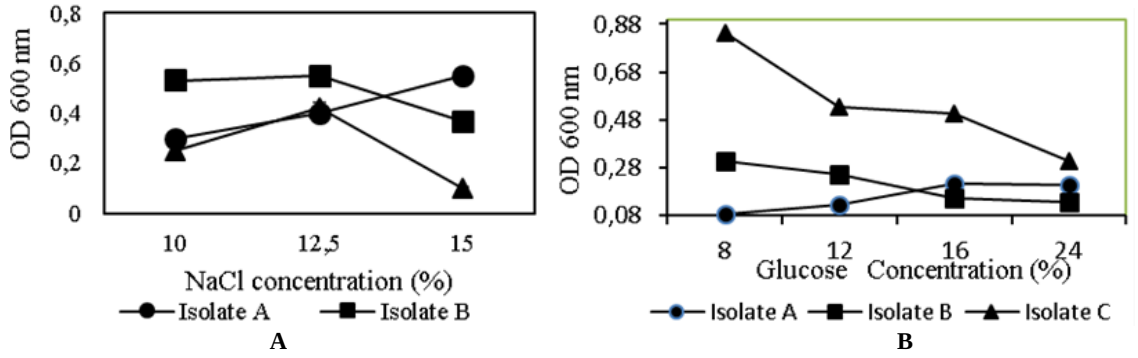


Figure 3. Growth of the metal-resistant bacteria under different NaCl (A) and glucose (B) concentrations

Table 3. Bacterial strains identified by 16s rRNA sequencing

Isolate	Phylum	Nearest phylogenetic neighbour (Accession no.)	Affiliation (Accession no.)	Similarity (%)
A	Proteobacteria	*Pseudomonas asiatica* strain CWC NVN (MK836042)	*Pseudomonas asiatica* strain A (MW304002)	100.0
B	Actinobacteria	*Sphingobacterium caeni* strain LH6 (MG786758)	*Sphingobacterium caeni* strain B (MW304003)	94.0
C	Proteobacteria	*Burkholderia cenocepacia* strain IST439 (LR798194)	*Burkholderia cenocepacia* strain C **(**MW304004)	97.4

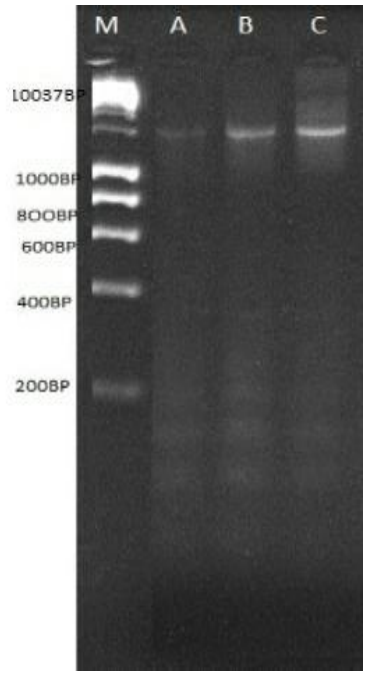


Figure 4. The agarose gel shows bands of the DNA fragments. Lane M represents the molecular ladder, while lanes A, B, and C represent the bands formed by the DNA of the isolates. These fall between 1000-10037 base pairs

Recall that the isolated bacterial strains produced some industrially relevant enzymes. Only 2% of the microbial population has been successfully investigated as enzyme sources (Demain 2000). The ability of soil microorganisms to synthesize enzymes aid in the biodegradation of environmental polymers and pollutants in the soil (Sun et al. 2014). A microorganism's ability to produce specific enzymes facilitates its heavy metal resistance ability (Thavamani et al. 2015). Cellulase is a cell wall degrading enzyme, and the extracellular secretion helps facilitate plant colonization by endophytes (Verma et al. 2001; Ma et al. 2015). Enzyme activities also assist in these organisms' heavy metal resistance ability and subsequently enhance the detoxification of heavy metals in contaminated soils (Thavamani et al. 2015). *Sphingobacterium athyrii* from decaying fern, produced both cellulase and xylanase (Cheng et al. 2019); however, *S. caeni* strain B did not secrete xylanase. Of special interest are the enzymes produced by the halophilic *P. asiatica* strain A. Halophilic bacteria are important sources of novel hydrolytic enzymes and are often extremozymes (Moreno et al. 2013; Munawar and Engel 2013). Typically, extremozymes tolerate extreme conditions, e.g., high salt concentrations, high temperature, and organic solvents. The *P. asiatica* is a recently proposed species of *Pseudomonas*, which before this study has only been isolated in Japan and Myanmar (Tohya et al. 2019). Our work is the first account of a halophilic metal-resistant strain. Both halophilic/halotolerant, osmophilic and enzyme-producing render our isolates potentially useful in a wide range of biological processes.

Based on the current findings, the isolated bacterial strains can serve as candidates for bioremediation, biodegradation and plant growth in metal-polluted soils or saline environments. However, the extent of their capabilities can only be determined following well-designed detailed studies with the respective bacterial strains. In addition, there is also a need to study the capacity of these strains to produce halotolerant or halophilic hydrolases, which are useful for various industrial processes.

Bacterial antibiotic resistance

All three bacterial strains were resistant to ampicillin and ceporex (Table 4). Notably, the *B. cenocepacia* exhibited resistance against all ten antibiotics tested, while *S. caeni* was the most susceptible. Burkholderia species are significantly antibiotic resistant (Rhodes and Schweizer 2016). The *P. asiatica* and *S. caeni* strains were susceptible to tarivid, reflacine, augmentin, and ciproflox. Multiple antibiotic resistance in bacterial strains may be related to the soil heavy metals, the isolate's genetic makeup, and the factors influencing gene transfer between bacteria (Rhodes and Schweizer 2016; Sinegani and Younessi 2017). Only *P. asiatica* was susceptible to streptomycin; meanwhile, it showed intermediate activity to nalidixic acid, gentamycin, and septrin. The antibiotic resistance was further represented as single-resistance, co-resistance, and multiple resistance to capture the resistance to one, two or more antibiotics, respectively (Figure 6). It was hypothesized that exposing bacteria to heavy metal increases antibiotic tolerance (Sharma et al. 2000; Verma et al. 2001; Samanta et al. 2012).

Table 4. Antibiotic sensitivity of the heavy metal-resistant bacterial isolates

Drug (mcg)	*P. asiatica*	*S. caeni*	*B. cenocepacia*
Ampicillin (30)	R	R	R
Ceporex (10)	R	R	R
Tarivid (10)	S	S	R
Nalidixic acid (30)	I	S	R
Reflacine (10)	S	S	R
Gentamycin (10)	I	S	R
Augmentin (30)	S	S	R
Ciproflox (10)	S	S	R
Streptomycin (30)	S	R	R
Septrin (30)	I	S	R

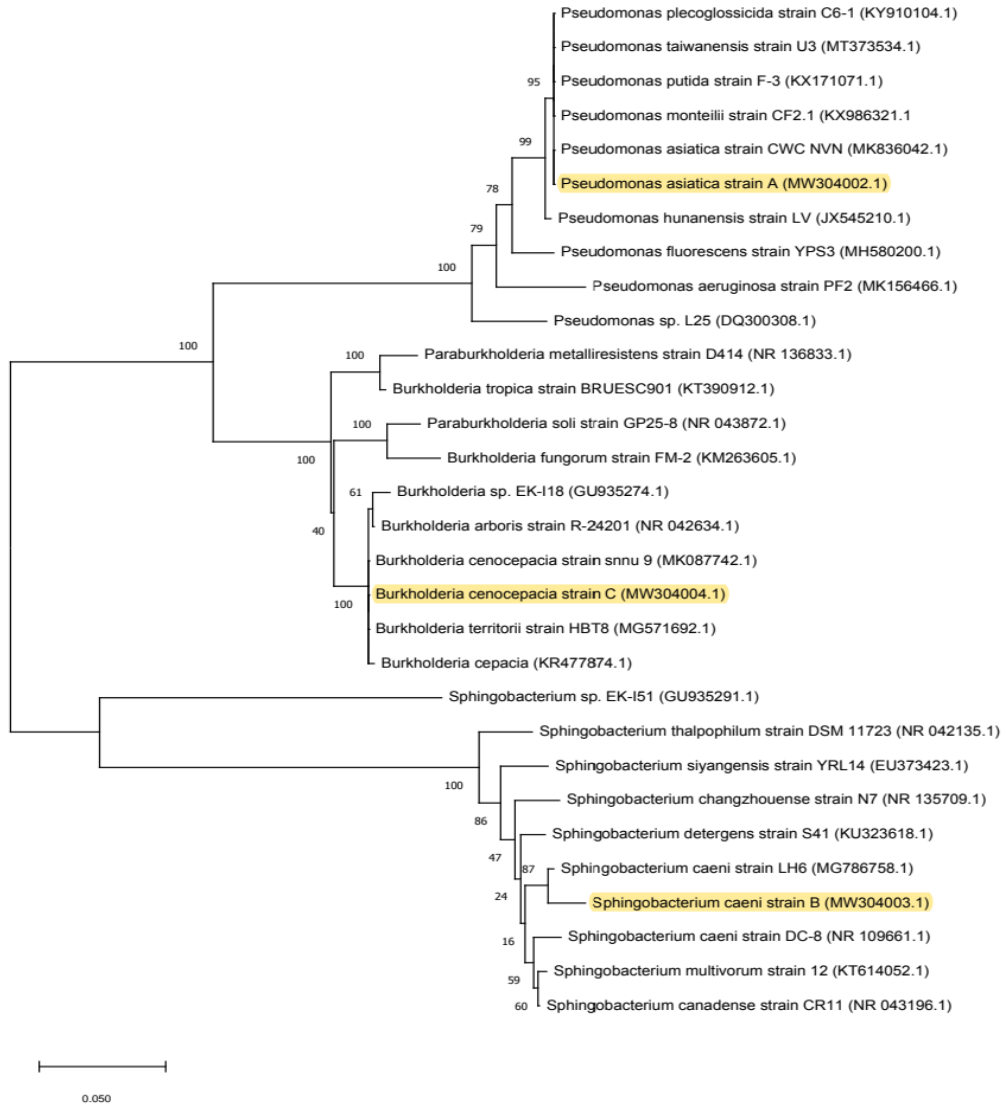


Figure 5. Phylogenetic relationships of the heavy metal-resistant isolates (*Pseudomonas asiatica* strain A, *Sphingobacterium caeni* strain B, *Burkholderia cenocepacia strain C*) to other closely related species

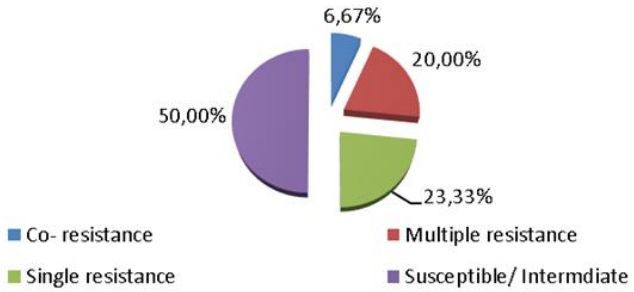


Figure 6. The percentage of antibiotic resistance of the bacterial strains. Letters in parenthesis indicate the sensitivity of the isolates to the antibiotic, and are represented as R: Resistant, I: Intermediate, and S: Susceptible

In conclusion, *P. asiatica* strain A, *S. caeni* strain B, and *B. cenocepacia* strain C were isolated from soils obtained from the metal dumpsite. The isolated bacteria thrived in high concentrations of Cu, Cd and Pb. The *P. asiatica* was the most resistant to the tested metals. The bacterial strains were halotolerant/ halophilic and osmotolerant and exhibited wide degrees of resistance to different antibiotics. The *B. cenocepacia* was resistant to all the antibiotics tested. The properties of the isolated bacteria strains imply their potential as bioremediation agents and possible candidates for producing halotolerant and osmotolerant hydrolases such as cellulase, xylanase, amylases and proteases, enzymes that currently dominate the world enzyme market. Further studies are required to ascertain the scope of these strains for the biotechnological processes.

REFERENCES

Abd WM, Shahin, RR, Khater HA, Ali MA. 2013. Isolation and identification of heavy-metal resistant soil bacteria. Am-Eur J Agric Environ Sci 13 (6): 831-838. DOI: 10.21608/jssae.2013.51953.

Abo-Amer AE, Abu-Gharbia MA, Soltan E-S M, El-Raheem WMA. 2014. Isolation and molecular characterization of heavy metal-resistant *Azotobacter chroococcum* from agricultural soil and their potential application in bioremediation. Geomicrobiology 31 (7): 551-561. DOI: 10.1080/01490451.2013.850561.

Ahirwar KN, Gupta G, Singh R, Singh V. 2016. Isolation, identification and characterization of heavy metal resistant bacteria from industrial affected soil in Central India. Intl J Pure Appl Biosci 4 (6): 88-93. DOI: 10.18782/2320-7051.2424.

Aka RJN, Babalola OO. 2017. Identification and characterization of cr-, cd-, and ni-tolerant bacteria isolated from mine tailings. Bioremed J 21 (1): 1-19. DOI: 10.1080/10889868.2017.1282933.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25 (17): 3389-3402. DOI: 10.1093/nar/25.17.3389.

Arora NK, Fatima T, Mishra J, Mishra I, Verma S, Verma R, Verma M, Bhattacharya A, Verma P, Mishra P, Bharti C. 2020. Halo-tolerant plant growth promoting *Rhizobacteria* for improving productivity and remediation of saline soils. J Adv Res 26: 69. DOI: 10.1016/j.jare.2020.07.003.

Bauer AW, Kirby WM, Sherris JC, Turck M. 1966. Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol 45 (4): 493-496. DOI: 10.1093/ajcp/45.4_ts.493.

Chellaiah E. 2018. Cadmium (heavy metals) bioremediation by *Pseudomonas aeruginosa*: A minireview. Appl Water Sci 8: 154. DOI: 10.1007/s13201-018-0796-5.

Cheng JF, Guo JX, Bian YN, Chen Z, Li CL, Li XD, Li YH. 2019. *Sphingobacterium athyrii* sp. nov., a cellulose- and xylan-degrading bacterium isolated from a decaying fern (*Athyrium wallichianum* Ching). Intl J Syst Evol 69 (3): 752-760. DOI: 10.1099/ijsem.0.003231.

Chihomvu P, Stegmann P, Pillay M. 2014. Identification and characterization of heavy metal resistant bacteria from the Klip river. Intl J Agric Food Vet Agric Eng 8 (11): 1107- 1117.

Chouari R, Paslier DL, Daegelen P, Ginestet P, Weissenbach J, Sghir A. 2005. Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. Environ Microbiol 7: 1104-1115. DOI: 10.1111/j.1462-2920.2005.00795.x.

Demain AL. 2000. Microbial biotechnology. Trends Biotechnol 18: 26-31. DOI: 10.1016/S0167-7799(99)01400-6.

Feng H, Zeng Y, Huang Y. 2014. S*phingobacterium paludis* sp. nov., isolated from wetland soil. Intl J Syst Evol 64 (10): DOI: 10.1099/ijs.0.064915-0.

Frostgard A, Baath E, Tunlid A. 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol Biochem 25 (6): 723-730. DOI: 10.1016/0038-0717(93)90113-P.

Gautam PK, Gautam RK, Banerjee S, Chattopadhyaya MC, Pandey JD. 2016. Heavy metals in the environment: Fate, transport, toxicity and remediation technologies. In: Pathania D (eds). Heavy Metals: Sources, Toxicity and Remediation Techniques. Nava Science Publishers, Hauppauge, New York.

Gurave NA, Korde VV, Dhas SS, Disale M. 2015. Isolation and identification of heavy metal resistant bacteria from petroleum soil of Loni, Ahmednagar. Eur J Exp Biol 5 (12): 6-11.

Hall CM, Busch JD, Shippy K, Allender CJ, Kaestli M, Mayo M, Sahl JW, Schupp JM, Colman RE, Keim P, Currie BJ, Wagner DM. 2015. Diverse *Burkholderia* species isolated from soils in the Southern United States with no evidence of *B. pseudomallei.* PLoS One 10 (11): e0143254. DOI: 10.1371/journal.pone.0143254.

Hashim MA, Mukhopadhyay S, Sahu JN, Sengupta B. 2011. Remediation technologies for heavy metal contaminated groundwater. J Environ Manag 92: 2355-2388. DOI: 10.1016/j.jenvman.2011.06.009.

Hussein H, Moawad H, Farag S. 2004. Isolation and characterization of *pseudomonas* resistant to heavy metal contaminants. Arabian J Biotechnol 7 (1): 13-22. DOI: 10.2225/vol7-issue1-fulltext-2.

Irawati W, Riak S, Sopiah N, Sulistia S. 2017. Heavy metal tolerance in indigenous bacteria isolated from the industrial sewage in Kemisan River, Tangerang, Banten, Indonesia. Biodiversitas 18: 1481-1486. DOI: 10.13057/biodiv/d180426.

Irawati W, Winoto SE, Kusumawati L, Pinontoan R. 2021. Indigenous multiresistant bacteria of IrC4 isolated from Indonesia as a heavy metal bioremediation agent. Biodiversitas 22: 3349-3355. DOI: 10.13057/biodiv/d220641.

Irwin JA. 2020. Overview of extremophiles and their food and medical applications. In: Salwan R, Sharma V (eds). Physiological and Biotechnological Aspects of Extremophiles. Academic Press, Cambridge. DOI: 10.1016/B978-0-12-818322-9.00006-X.

Jiang CY, Sheng XF, Qian M, Wang QY. 2008. Isolation and characterization of a heavy metal-resistant *Burkholderiasp*. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. Chemosphere 72 (2): 157-64. DOI: 10.1016/j. *Chemosphere*.2008.02.006.

Jiang J, Pan C, Xiao A, Yang X, Zhang G. 2017. Isolation, identification, and environmental adaptability of heavy metal- resistant bacteria from ramie rhizosphere soil around mine refinery. Biotech 7: 5. DOI: 10.1007/s13205-017-0603-2.

Juhaeti T, Syarif F, Hidayati N. 2004. Inventarization of potential plant for phytoremediation on degraded land and water mined. Biodiversitas 6: 131-133. DOI: 10.13057/biodiv/d060106.

Kathiravan MN, Karthick R, Muthukumar K. 2011. Ex situ bioremediation of Cr (VI) contaminated soil by *Bacillus sp.*: batch and continuous studies. Chem Eng J 169: 107-115. DOI: 10.1016/j.cej.2011.02.060.

Khosro I, Nadiya J, Fataneh P, Golnaz, R, Jamileh F. 2013. Heavy metal resistance by bacterial strains. Sch Res Libr 2: 60-63.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120. DOI: 10.1007/BF01731581.

Korcan SE, Konuk M, Erdoğmuş SF. 2015. Beneficial usages of halophilic microorganisms. In: Maheshwari D, Saraf M (eds). Halophiles. Sustainable Development and Biodiversity, Vol 6. Springer, Cham, Switzerland. DOI: 10.1007/978-3-319-14595-2_10.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35: 1547-1549. DOI: 10.1093/molbev/msy096.

Liu XX, Hu X, Cao Y, Pang WJ, Huang JY, Guo P, Huang L. 2019. Biodegradation of Phenanthrene and heavy metal removal by acid-tolerant *Burkholderia fungorum* FM-2. Front Microbiol 10: 408. DOI: 10.3389/fmicb.2019.00408.

Liu Y, Tie B, Li Y, Lei M, Wei X, Liu X, Du H. 2018. Inoculation of soil with cadmium-resistant bacterium *Delftia* sp. B9 reduces cadmium accumulation in rice (*Oryza sativa* L.) grains. Ecotoxicol Environ Safe 163: 223-229. DOI: 10.1016/j.ecoenv.2018.07.081.

Ma Y, Oliveira RS, Nai F, Rajkumar M, Luo Y, Rocha I, Freitas H. 2015. The hyperaccumulator *Sedum plumbizincicola* harbors metal-resistant endophytic bacteria that improve its phytoextraction capacity in multi-metal contaminated soil. J Environ Manag 156: 62-69. DOI: 10.1016/j.jenvman.2015.03.024.

Mendez MO, Nielson JW, Maier RM. 2008. Characterization of a bacterial community in an abandoned semiarid lead-zinc mine tailing site. Appl Environ Microbiol 74: 3899-3907. DOI: 10.1128/AEM.02883-07.

Mohamed RM, Abor-Amer AE. 2012. Isolation and characterization of heavy-metal resistant microbes from roadside soil and phylloplane. J Basic Microbiol 52: 53-65. DOI: 10.1002/jobm.201100133.

Moreno M, Perez D, Garcia MT, Mellado E. 2013. Halophilic bacteria as a source of novel hydrolytic enzymes. Life (Basel) 3 (1): 38-51. DOI: 10.3390/life3010038.

Munawar N, Engel PC. 2013. Halophilic enzymes: Characteristics, structural adaptation and potential applications for biocatalysis. Curr Biotechnol 2 (4): 15-16. DOI : 10.2174/1872208311307666003 3.

Neethu CS, Mujeeb-Rahiman KM, Saramma AV, Mohamed-Hatha AA. 2015. Heavy metal resistance in Gram-negative bacteria isolated from Kongsfjord, Arctic. Canad J Microbiol 61 (6): 429-435. DOI: 10.1139/cjm-2014-0803.

Nguyen TT, Lama S, Ainala SK, Sankaranarayanan M, Chauhan AS, Kim JR, Park S. 2021. Development of *Pseudomonas asiatica* as a host for the production of 3-hydroxypropionic acid from glycerol, Biores Technol 329: 124867. DOI: 10.1016/j.biortech.2021.124867.

Noura, Salih KM, Jusuf NH, Hamid AA, Yusoff WM. 2009. High prevalence of *Pseudomonas* species in soil samples from Ternate Island-Indonesia. Pak J Biol Sci 12 (14): 1036-1040. DOI: 10.3923/pjbs.2009.1036.1040.

Pacwa-Plociniczak M, Plociniczak T, Yu D, Kurola JM, Sinkkonen A, Piotrowska-Seget Z, Romantschuk M. 2018. Effect of *Silene vulgaris* and heavy metal pollution on the microbial diversity in long-term contaminated soil. Water Air Soil Pollut 229: 13. DOI: 10.1007/s11270-017-3655-3.

Patel S, Saraf M. 2015. Perspectives and application of halophilic enzymes. In: Maheshwari D., Saraf M (eds). Halophiles. Sustainable Development and Biodiversity, Vol 6. Springer, Cham, Switzerland. DOI: 10.1007/978-3-319-14595-2_15.

Raja EC, Selvam GS, Omine K. 2009. Isolation, Identification and Characterization of Heavy Metal Resistant Bacteria from Sewage. International Joint Symposium on Geodisaster Prevention & Geoenvironment in Asia, Fukuoka.

Rhodes KA, Schweizer HP. 2016. Antibiotic resistance in *Burkholderia* species. Drug Resist Updat 28: 82-90. DOI: 10.1016/j.drup.2016.07.003.

Samanta A, Bera P, Khatun M, Sinha C, Pal P, Lalee A, Mandal A. 2012. An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain *Bacillus sp*. isolated from municipal waste. J Microbiol Biotechnol Res 2 (1): 178-189.

Sharma KP, Frenkel A, Balkwill L D. 2000. A new *Klebsiella planticola* strain (cd-1) grows an aerobically at high cadmium concentrations and precipitates cadmium sulphide. Appl Environ Microbiol 66: 3083-3087. DOI: 10.1128/AEM.66.7.3083-3087.2000.

Sheng F, Xia J, Jiang C,Yanhe L, Meng Q. 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and accumulation of rape. Environ Pollut 156: 1164-1170. DOI: 10.1016/j.envpol.2008.04.007.

Sinegani AAS, Younessi N. 2017. Antibiotic resistance of bacteria isolated from heavy metal-polluted soils with different land uses. J Glob Antimicrob Resist 10: 247-255. DOI: 10.1016/j.jgar.2017.05.012.

Singh P, Jain K, Desai C, Tiwari O, Madamwar D. 2019. Microbial Community Dynamics of Extremophiles/Extreme Environment. In: Das S, Dash HR (eds). Microbial Diversity in the Genomic Era. Academic Press, Cambridge. DOI: 10.1016/B978-0-12-814849-5.00018-6.

Singh V, Chauhan PK, Kanta R, Dhewa T, Kumar V. 2010. Isolation and characterization of *Pseudomonas* resistant to heavy metals contaminants. Intl J Pharm Sci Rev Res 3 (2): 164-167.

Sulowicz S, Plociniczak T, Piotrowska-Seget Z, Kozdroj J. 2011. Significance of birch and bushgrass for establishment of microbial heteroptrophic community in a metal-mine spoil heap. Water Air Soil Pollut 214: 205-218. DOI: 10.1007/s11270-010-0417-x.

Sun J, Zhang Q, Zhou J, Wei QP. 2014. Il- lumina Amplicon Sequencing of 16S rRNA tag reveals bacterial community development in the rhizosphere of apple nurseries at a replant disease site and a new planting site. Plos One 9 (10): 111744. DOI: 10.1371/journal.pone.0111744.

Sun LN, Zhang J, Chen Q, He J, Li SP. 2013. *Sphingobacterium caeni* sp. nov., isolated from activated sludge. Intl J Syst Evol Microbiol 63 (6): 2260-2264. DOI: 10.1099/ijs.0.046987-0.

Thavamani P, Megharaj M, Naidu R. 2015. Metal-tolerant PAH degrading bacteria: Development of suitable test medium and effect of cadmium and its availability on PAH biodegradation. Environ Sc Pollut Res 22: 8957-8968. DOI: 10.1007/s11356-013-1850-3.

Tohya M, Watanabe S, Teramoto K, Uechi K, Tada T, Kuwahara-Arai K, Kinjo T, Maeda S, Nakasone I, Zaw NN, Mya S, Zan KN, Tin HH, Fujita J, Kirikae T. 2019. *Pseudomonas asiatica* sp. nov., isolated from hospitalized patients in Japan and Myanmar. Intl J Syst Evol Microbiol 69 (5):1361-1368. DOI: 10.1099/ijsem.0.003316.

Torres Perez MP, Deimer VR, Alexander PC. 2019. Bioremediation of mercury and nickel by endophytic bacteria of aquatic macrophytes. Rev Colomb Biotecnol 21 (2): 36-44. DOI: 10.15446/rev.colomb.biote.v21n2.79975.

Veras BO, dos Dantos YQ, Diniz KM, Carelli GS, dos Santos EA. 2018. Screening of protease, cellulase, amylase and xylanase from the salt-tolerant and thermostable marine *Bacillus subtilis* strain SR60. F1000 Res 7: 1704. DOI: 10.12688/f1000research.16542.1.

Verma T, Srinath T, Gadpayle RU, Ramtake PW, Hans RK, Garg SK. 2001. Chromate tolerant bacteria isolated from tannery effluent. Bioresour Technol 78: 31-35. DOI: 10.1016/S0960-8524(00)00168-1.

Waditee-Sirisattha T, Kageyama H, Takabe T. 2016. Halophilic microorganism resources and their applications in industrial and environmental biotechnology. AIMS Microbiol 2 (1): 42-54. DOI: 10.3934/microbiol.2016.1.42.

Wang J, Chen C. 2009. Biosorbents for heavy metals removal and their future. Biotechnol Adv 27: 195-226. DOI: 10.1016/j.biotechadv.2008.11.002.

Zulkali MM, Ahmad AL, Norulakmal NH. 2006. *Oryza sativa* Husk as heavy metal adsorbent: optimization with lead as model solution. Bioresour Technol 97: 21-25. DOI: 10.1016/j.biortech.2005.02.007.

ASIAN J TROP BIOTECHNOL
Volume 19, Number 1, June 2022
Pages: 20-27

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c190104

Effect of alkaline delignification on physico-chemical and combustion properties of bean chaff briquette

ADEGOKE IDOWU ABIMBOLA[1,♥], IGE AYODEJI RAPHEAL[2], BAMISAYE ABAYOMI[3], ELINGE COSMOS MOKI[2], OLUBUNMI O. AYODELE[4]

[1]Department of Forestry, College of Agriculture, Fisheries and Forestry, Fiji National University. Kings Road, Koronivia, Fiji Island. Tel.: +679-347-9200 Ext: 5001, ♥email: adegokeabimbola4u@yahoo.com
[2]Pure and Applied Chemistry Department, Kebbi State University of Science and Technology. Aliero, Nigeria
[3]Department of Chemistry, Faculty of Basic Medical and Applied Sciences, Lead City University. Ibadan, Nigeria
[4]Department of Nanoscience, School of Nano science and Nano engineering, University of North Carolina. Chapel Hill, North Carolina, USA

Manuscript received: 9 February 2022. Revision accepted: 16 June 2022.

Abstract. *Abimbola AI, Rapheal IA, Abayomi B, Moki EC, Ayodele OO. 2021. Effect of alkaline delignification on physico-chemical and combustion properties of bean chaff briquette. Asian J Trop Biotechnol 19: 20-27.* Adopting lignocellulose-rich agro-waste materials for briquette production could be regarded as a better alternative energy source and also helps to ameliorate the challenges associated with deforestation and agro-waste disposal. This study examined the effects of alkali pretreatment on briquettes produced from bean chaffs. The proximate analysis, Energy Dispersive X-ray Fluorescence (EDXRF), Fourier-Transform Infrared (FTIR) analysis, Scanning electron microscopy (SEM), physical analysis and combustion properties were determined for the briquettes produced. The mean moisture content of 3.52±0.10% and 5.81±0.01% were recorded for Treated Bean Chaff (TBC) and Untreated Bean Chaff (UBC) briquettes, respectively. A high heating value of 24.18±0.12 MJ/kg was recorded for TBC compared to 21.12±0.01 MJ/kg of UBC briquette samples. Furthermore, it was observed that alkali pretreatment reduced the percentage of Potential Toxic Element (PTE) concentration in the treated sample, as shown by EDXRF. The FTIR results reveal surface modification in the fiber matrix shown by the C-O band shift from 1013 cm^{-1} observed in TBC to 1010 cm^{-1} recorded in UBC. In contrast, SEM shows clear disruption in the biomass matrix due to the alkali pretreatment process. The findings of this study show that the alkaline pretreated bean chaff briquettes have a great potential to be used as biomass fuel.

Keywords: Agro-waste, bean chaff, biomass, briquette, lignocellulose

INTRODUCTION

Bean production occurs in many countries, and Nigeria is one of the world's largest producers and consumers. Common bean also has the economic and environmental benefit of associating with nitrogen-fixing bacteria, which gives an advantage to fixing atmospheric nitrogen and leaving phosphorous (P) for plant growth. It is widely appreciated in developing countries for its affordability and long storage life. It is the most important crop for food security and wealth creation (Hordofa and Etisa 2019).

The utilization of agricultural and forest wastes as biomass is being progressively more studied and could serve as an alternative to fossil fuels and related problems (Fernandes et al. 2013; Amirta et al. 2016). But conversely, it is not easy to handle, transport, store, and use biomass in its original shape due to some factors, including high moisture content, irregular shape and sizes, and low bulk density (Karunanithy et al. 2012). Therefore, it is important to improve energy efficiency and technological innovation for biomass to be a notable impact as fuel.

The application of briquetting technology is one of the promising technology solutions to these problems. According to Wilaipon (2007), briquetting can be defined as a densification process for improving raw materials' handling qualities and enhancing the biomass's volumetric calorific value. In addition, the briquetting technology improves physical and combustion characteristics. The production of briquettes from raw biomass has been extensively studied (Husain et al. 2002; Ndiema et al. 2002).

Modifying the surface of biomass materials utilizing alkaline pretreatment is proficient and is usually done to advance adhesion between the biomass materials and improve the quality properties of the briquette (Mahalingam et al. 2019; Elinge et al. 2020). The briquette production process can be adjusted to improve the quality of the briquette through alkaline pretreatment of the agricultural biomass. Several researchers have generated briquettes from agricultural wastes, but few studies on the alkaline pretreatment of agricultural biomass for briquette production (Elinge et al. 2020; Oyibo et al. 2020; Bamisaye and Rapheal 2021).

To the best of our knowledge, there is no account to study the utilization of beans chaff for the production of biofuels. Therefore, there is a need to investigate the influence of alkaline pretreatment on the proximate, chemical, physical, and combustion properties of the briquette produced from beans chaff.

MATERIAL AND METHODS

Materials

The bean chaff used for this study, as shown in Figure 1, was sourced from Gashua, Yobe State, Nigeria. After collection, it was washed with tap water to remove impurities and dried in the oven at 105ºC for 24 hours. The dried biomass was sieved with a medium size, 2 mm particle and stored in airtight containers until further use.

Alkaline pretreatment

The bean chaff surface was treated with an alkaline solution. Half a cup of beans chaff was treated with 100 ml solution at 3% concentration, and soaking was done for 3 hours at room temperature. After soaking, the sample was filtered and washed with distilled water five times to get pH of 7.0. The washed residues were dried in an oven at 105ºC.

Lignocellulosic composition analysis

Analysis of lignin and cellulose was done according to the method of Mahyati et al. (2013).

The mixture containing 1 g of dry sample (a) and 150 ml of demineralized water was heated in a bathtub at a temperature of 100ºC per hour. The mixture was filtered, and the residue was washed with warm water (300 mL). The residue was dried in the oven until the weight was constant (b). Next, the residue was mixed with 150 mL of 1 N H_2SO_4 and heated in an oil bath at 100ºC for 1 hour. The mixture was dissolved and washed with 300 mL of water, demineralized, and then the residue was dried (c). The remaining residue is soaked in 10 milliliters of 72% H_2SO_4 at room temperature for 4 hours. After that 150 mL of 1 N H_2SO_4 was injected into the mixture and re-injected into the oil bath for an hour. The solid was dissolved with 400 mL of demineralized water, heated in the oven at 105ºC and measured until constant weight (d). Finally, the solid was heated to ashes and measured (e). The proportion of hemicellulose, cellulose and lignin was calculated as follows:

$$\%\ hemicellulose = \frac{(c-b)}{a} \times 100\% \ldots\ldots 1$$

$$\%\ cellulose = \frac{(d-c)}{a} \times 100\% \ldots\ldots 2$$

$$\%\ lignin = \frac{(e-d)}{a} \times 100\% \ldots\ldots 3$$

Briquette production

Briquettes are produced using a briquetting machine that operates on a 10-ton hydraulic jack that attaches to a cylindrical mold chamber that compresses the slurry. The length and width of the briquettes are 94 mm and 61 mm, respectively. Processed cassava starch was purchased from Gashua market, Yobe State, and was used as a binder. The untreated bean chaff was measured, mixed in a ratio of 4: 1, thoroughly mixed with water to form a slurry and fed into the molds to form briquettes and the briquettes were dried in the sun for two weeks.

Determination of physico-chemical and combustion properties of briquettes

Ash content, moisture content, fixed carbon, and volatile matter of the dried matter and briquette sample were determined according to the specifications ASTM D-3172 (ASTMS 2021). The calorific system of briquette photographs is obtained using the LECO AC 350 bomb calorimeter in the system design (ASTMS 2019). Density was determined by DIN 52182 and specifications 51731 published elsewhere (Krizan et al. 2009). The compressive strength capacity of the briquette sample was determined using the ELE tritest 50 compression machine according to specifications D 3173-878 (Wilaipon 2007). The boiling water test was performed by recording the time set for the maximum amount of 500 g of briquette to start boiling 500 ml of water at various meeting conditions.

Elemental composition

Based on the results of the proximate evaluation, the primary sources of common elements such as carbon (C), hydrogen (H), and oxygen (O) for briquettes were estimated using Equations (1), (2), and (3), respectively. These were determined at an estimate of 95% confidence level (Jigisha et al. 2007):

C = 0.637 F c + 0.455 V m ..... (1)
H = 0.052 F c + 0.062 V m ..... (2)
O = 0.304 F c + 0.476 V m ..... (3)

Figure 1. A. Bean chaffs; B. Manual piston briquetting machine, C. Untreated briquette samples, D. Treated briquette samples

Energy dispersive x-ray fluorescence (EDXRF)

EDXRF is a non-Functional tool for the measurable and qualitative determination of main and trace elements in a wide range of sample types (Jyothsna et al. 2020). The EDXRF measurement was performed with the following experimental parameters values: side window tube, high voltage tube: max. 30 kV; emission current: max. 1 mA; power: max. 9 W; air cooled (Ion et al. 2007). The Capacities of X-Ray emitted are detected using Si(Li) and processed by a high Pulse Analysis (Jyothsna et al. 2020).

FTIR (Fourier Transform Infrared Spectroscopy)

Infrared bands of alkali-treated and untreated bean chaffs were measured on AVATAR 330 Fourier Transform Infrared (FT-IR) Spectrophotometer.

Scanning electron microscopy

The analysis of the microstructure of alkali-treated and untreated bean chaffs were evaluated by Scanning Electron Microscopy (SEM). The samples were first transformed into capsules, coated with Palladium (Pd) at 30 mA, and analyzed in a JEOLJFC-5510LV Scanning Electron Microscope.

Statistical analysis

The sum of all the parameters analyzed was calculated by two T-tests (Statistical significance determined using the Holm-Sidak method, with alpha=5.000%.) using Graph Pad Prism® (Version 6.04) and results were presented as mean ± SEM.

RESULTS AND DISCUSSION

Alkaline pretreatment is one major practice that helps improve biofuel quality through bond loosening and fractionation of the biomass matrix, thereby bringing about an effective separation between the lignin and carbohydrates (Zhao et al. 2008). This increases the hemicellulose and cellulosic contents of the biomass. Also, it helps to increase the internal surface area of biomass through adequate disruption of lignin structure and delignification and decreases the degree of crystallinity and polymerization of the potential solid fuel (Zhao et al. 2010; Conde-Mejia 2012). The observed result of the lignocellulosic contents of the untreated (UBC) and alkaline treated bean chaff (TBC) was presented in Figure 2. This shows that alkali pretreatment increases the cellulose and hemicellulose contents of the treated samples. Recorded mean values of 43.20±0.30% (TBC) and 9±0.10% (TBC) were observed compared to the untreated raw bean chaff with a mean value of 40.60±0.02% (UBC) and 8±0.43% (UBC) for the cellulose and hemicellulose contents respectively. Furthermore, the lignin contents of the treated sample were observed to reduce compared to the untreated, as shown in Figure 2. The recorded mean lignin contents of UBC and TBC were 10±0.01 and 8±0.01%, respectively. However, suggested that alkali-pretreatment of TBC results in delignification and increases the cellulose and hemicellulose contents of the biomass sample (Zhao et al. 2010; Bensah and Mensah 2013).

The proximate composition analysis is a determinant of the potentials hidden in any biomass samples and as such, its report helps to know if a supposed biomass sample can be harnessed as an alternative source of energy. Figure 3A shows the proximate composition of the untreated and alkaline treated bean chaff before briquetting, while Figure 3B shows the proximate composition of the untreated and alkaline treated bean chaff briquettes.

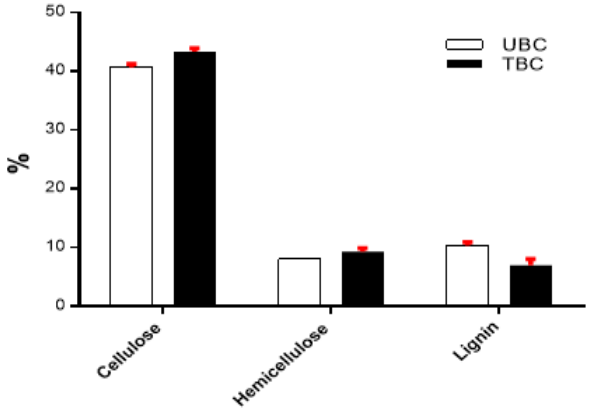


Figure 2. Lignocellulosic contents of the untreated and alkaline treated bean chaff

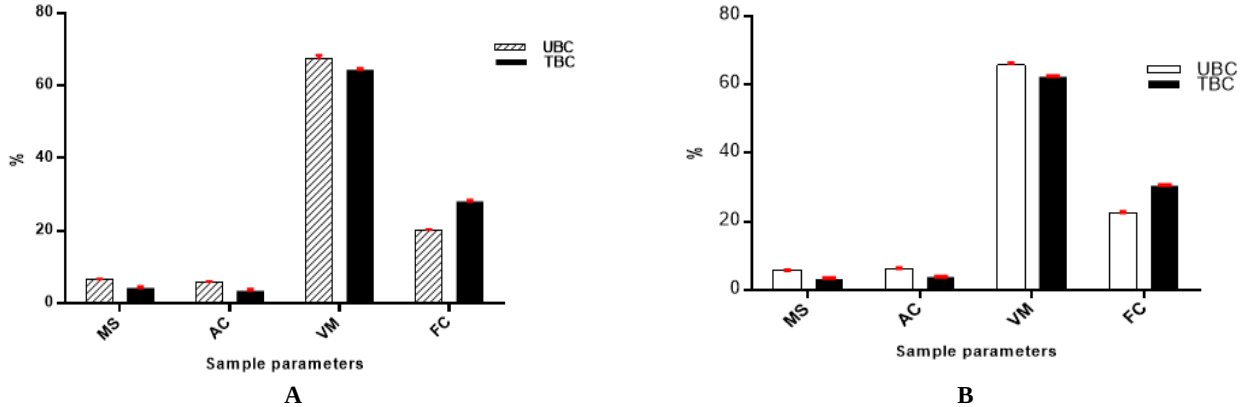


Figure 3. A. Proximate composition of the untreated and alkaline treated bean chaff before briquetting, B. Proximate composition of the untreated and alkaline treated bean chaff briquettes

The combustion strength of any biofuel determines the probability of its usability of such for its intended purpose. Such moisture content is the key to this because it measures the degree of dryness or wetness of the briquette (Demirbas 2013; Abayomi et al. 2021) and the shelf life of a briquette sample (Rapheal et al. 2018). Figure 3A shows a mean value of 6.52±0.02% recorded for UBC and 4.41±0.10% for TBC at $p<0.05$ before briquetting. However, a reduction in the moisture content value of the briquette was observed to be smaller at a mean value of 3.52±0.10 % for TBC briquette compared to 5.81±0.01 % observed in the UBC briquette samples. Although, all the samples- both treated and untreated raw and briquette chaff- were observed to be within the acceptable range value of 10-15% moisture content reported by (Maciejewska et al. 2006). This study's result is lower than 9.35±0.17% recorded for treated banana leaves (Bamisaye and Rapheal 2021) and 8.08%, as reported by Deepak et al. (2019). This shows that both the treated and the untreated raw and briquette samples can be considered good biomass and can be used as a solid fuel. The treated briquette sample (TBC briquette) is the best alternative due to its lowest moisture content of 3.52±0.10 %, as shown in Figure 3B, and will have a positive effect on the stored energy value of the briquette.

Determining the ash content of any combustible solid fuel is very important because it gives information about the potential pollution that could result from burning such material. It is a good pointer to environmental pollution management. The ash content and the EDXRF are very important to prevent the potential public health challenges resulting from the usability of any fuel for industrial or domestic purposes. Reports have shown that the ash content value of any biofuel should not exceed 20 % and must be between 5 to 20%. This shows that a low ash content value (that is, <20%) briquette is better quality. Figure 3 shows a mean ash content value of 6.20±0.03% for the UBC of briquette compared to 3.93±0.20% TBC briquette. However, the raw TBC sample recorded a mean value of 3.10±0.01%, as shown in Figure 3B. Upon comparing the percentage ash content of TBC of the raw chaff and briquette, the result shows that briquetting (densification) does not literarily have a significant effect on the ash content value in this sample. However, alkali treatment does, with a good observable difference between the treated and untreated briquette and chaff sample, as shown in Figure 3. This suggests that the treated samples (TBC) raw chaff and briquette with lower ash content value, as shown in Figure 4, are considered to be of better quality than UBC for both the raw chaff and the briquette. The percentage ash content value of this study is lower compared with the observed to be lower when compared with the ash contents of 13.17±0.13% and 15.71±0.29%, which were recorded for both treated starch-bonded and paper-bonded banana leaves, respectively (Bamisaye and Rapheal 2021).

An observable difference in the volatile matter content values of 64.50±0.02% and 67.40±0.30% were recorded for both UBC and TBC at $P<0.05$, respectively, for the raw chaff, as shown in Figure 2. Likewise, for the briquettes sample, a recorded mean values of 62.29±0.01% (TBC) and 66.25±0.05% (UBC). Reports have shown that the rate of combustion and the stored energy released by a solid fuel could be affected by the percentage of the volatile matter content of such biomass (Maninder et al. 2012; Rapheal et al. 2018). This shows that the higher the volatile content values of solid fuel, the faster its ignition rate and the faster it would burn off. This, therefore, shows that the UBC of the raw chaff will burn off faster compared with the TBC briquette. Aside from alkali pretreatment, densification could also be observed to improve the TBC's combustibility property, as shown in Figure 3A, with 64.50±0.02% recorded for TBC chaff and 62.29±0.01 for TBC after briquetting.

There is a highly significant difference in the observed fixed carbon content of the treated and untreated biomass samples; for both the raw chaff and its briquette counterpart. The fixed carbon content of TBC after briquetting was observed to be the highest at a mean value of 30.26±0.01% compared to 21.74±0.12% for UBC for the briquette sample, as shown in Figure 3B. Densification and alkali pretreatment was observed to have improved the quality of the FC of the biofuel. The study, however, shows that the briquette with the highest FC value will have a correspondingly high rate of energy release and a lower time of cooking compared to the ones with low FC value, which are the UBC of both untreated biomass of the raw chaffs and the briquettes samples as shown in Figure 3.

The physical characteristics and combustion properties of untreated and alkaline-treated bean chaff briquettes, including the density, compressive strength, combustibility test and the time taken to burn to ashes, were presented in Table 1. The density and compressive strength are determinant parameters for the compactness of solid biofuel. In addition, they determine the shelf life of briquettes. This means that a solid fuel with a considerably high density or compact value will last longer in storage which can aid its transportability without any observable compromise in the integrity of the stored energy value of the biofuel. The recorded high density and compressive strength values of 0.71±0.10 N/mm^2 and 1.11±0.20 g/cm^3 in the alkali-treated (TBC) sample compared to the UBC value shown in Table 1 can be attributed to the reduction in diameter of the fiber matrices of the biomass samples. This corroborates the result obtained in Figure 2. Thereby confirming that delignification, which is the disruption and restructuring of the biomass matrices, has occurred, and the impact of these could be observed in the time taken to burn to ashes in which TBC recorded a value of 14.12±0.02 min compared to 12.40±0.14 min to boil 500 cm3 recorded for UBC. This, therefore, shows that alkaline treatment improved the quality of the solid biofuel produced from raw bean chaff through an observable increase in fixed carbon content, density, and calorific value, which are essential to the heat energy of a biofuel.

Table 1. Physical characteristic and combustion properties of untreated and alkaline treated bean chaff briquettes

Sample/parameters	UBC	TBC
Density (g/cm^3)	0.52±0.05	0.71±0.10
Compressive strength (N/mm^2)	0.72±0.03	1.11±0.20
Water quantity (cm^3)	500	500
Combustibility test (min)	6.21±0.40	4.57±0.40
Time taken to burn to ashes (min)	12.40±0.14	14.12±0.02

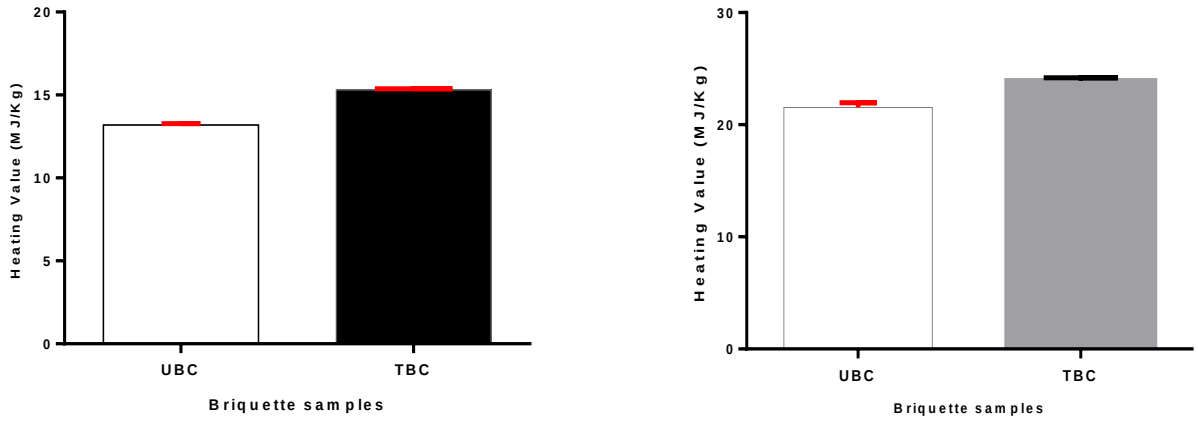
The amount of stored heat energy in the briquette sample is shown by the measure of its heat value, otherwise known as the calorific value (Santhebennur and Jogttappa 2012). The raw and briquette samples' heating values are presented in Figures 4A and 4B, respectively. Mean high heating values of 24.18±0.12 and 21.12±0.01 MJ/kg were recorded for both the TBC and UBC of the briquette samples shown in Figure 4B compared to the raw chaff with a low mean value of 15.41±0.10 (TBC) and 13.17±0.01 MJ/kg (UBC) shown in Figure 4B. The results are compared well with Brand and Jacintho's (2017) report, in which a calorific value of 17.98 MJ/kg was recorded, and Lubwama and Yiga (2017) recorded a value of 23 MJ/kg were recorded. This study showed an observable high significant difference between the heating value of the raw chaff and the briquette produced from bean chaff which is suggestive of being as a result of densification improved the heating capacity of a solid biofuel due to the compactness of the fiber matrices resulting to low porosity as a result of delignification.

The ultimate analysis of both UBC and TBC is presented in Table 2. The result indicated that all the analyzed parameters, including carbon, oxygen, and hydrogen contents, increased in the treated samples compared to the untreated, as shown in Table 2. However, the differences in the results of the obtained values of oxygen and hydrogen contents of UBC and TBC were minimal compared to the large difference in the mean value observed in the percentage carbon content of UBC and TBC. The recorded mean values of 43.60±0.02% were observed for carbon content in UBC compared to TBC with a mean value of 47.35±0.10%, which was higher than the untreated sample (Matali et al. 2016). Also, the ultimate analysis results were corroborated by the EDXRF results shown in Figure 4B compared to Figure 4A, in which the observed counts of carbon were observed to be slightly below 1000 counts in the UBC spectrum compared to the TBC spectrum in which carbon counts was observed to be above higher than 1000 counts (Figure 4B) This have an impact on the heating capacity and fixed contents of the biomass samples of the raw bean chaff prior briquetting and after briquette production as shown in Figure 3A and 3B, respectively.

Table 2. Ultimate analysis of untreated and alkaline treated bean chaff

Samples	C%	O%	H%
UBC	43.60±0.02	38.25±0.01	5.24±0.30
TBC	47.35±0.10	39.29±0.01	5.47±0.02

Note: Data are means of three replicates (n= 3) ± SEM using Graph Pad. Prism, t-test. UBC: Untreated Bean Chaff, TBC: Treated Bean Chaff



A **B**

Figure 4. A. Heating value untreated and alkaline treated bean chaff, B. Heating value untreated and alkaline treated bean chaff briquettes

Table 3. Elemental concentrations of untreated and alkaline treated bean chaff

Elements	K	Ca	Mg	Fe	Na	P	S	Pb	Sn	Si	Nb	Ba
UBC (%)	1.45	1.23	0.04	0.05	0.00	0.20	0.09	0.18	0.00	0.36	0.17	0.17
TBC (%)	0.63	1.10	0.05	0.05	0.29	0.18	0.07	0.40	0.07	0.65	0.05	0.27

Note: UBC: Untreated Bean Chaff, TBC: Treated Bean Chaff

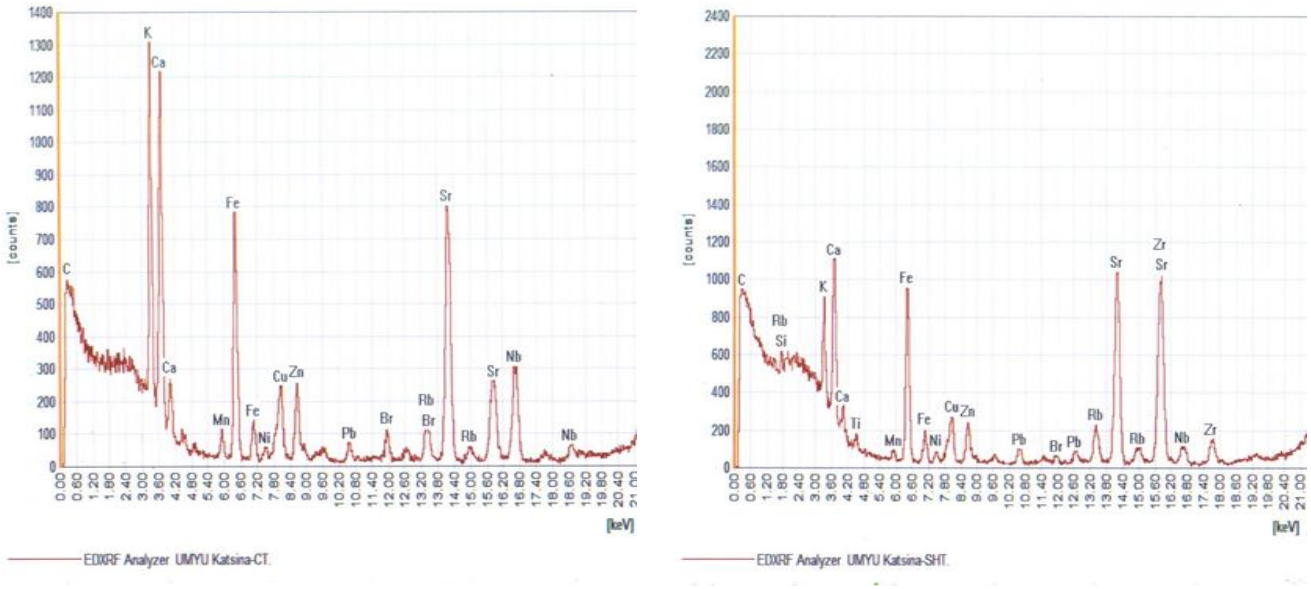


Figure 5. A. The EDXRF Spectrum of Untreated Bean Chaff (UBC), B. The EDXRF Spectrum of alkaline Treated Bean Chaff (TBC)

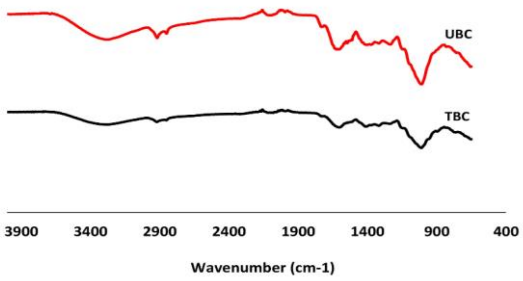


Figure 6. Showing the FTIR spectrum of untreated (UBC) and alkaline-treated bean chaff (TBC)

Table 4. Showing the FTIR vibrational stretch of untreated (UBC) and alkaline-treated bean chaff (TBC)

S/n	UBC (cm^{-1})	TBC (cm^{-1})	Assignment
1	3280	3268	O-H
2	2918	2922	Methylene Ass/Sym
3	2851	2851	Sp^3 stretch
5	1733	-	Aldehyde, C-O-H
5	1606	1601	NH_3 deformation
6	1010	1013	C-O
7	-	764	C-H, bending vibration

The EDXRF is one of the important analytical non-destructive multi-element analyses for determining trace or potential toxic elements (PTEs) in biomass samples. The major application of this technique in this work is to determine the amount of PTE at their trace level in a potential solid fuel. Thus, monitoring the rate of potential environmental pollution and health hazards that could arise from the continual usage of such fuel. This study shows that alkaline pretreatment improves the biomass's combustion properties and fuel quality, as shown in Table 3. On the other hand, the PTEs that are sulfur (s), lead (Pb), tin (Sn), and niobioun (Nb) tend to cause public health havoc; eye, skin, brain, and respiratory tract diseases (Ali et al. 2019), were observed to have been reduced drastically as shown in Table 3. Also, the carbon content, which determines the efficiency of combustibility and one of the potential parameters that determine the usage of any biomass for fuel purposes, was observed to have increased due to alkali pretreatment, as shown in Figure 5 for UBC and TBC, respectively.

The FTIR analysis was used to assess the surface modification and restructuring in both treated and untreated samples. The results of this analysis are presented in Table 4. The absorption band in the range of 3570-3200 cm^{-1}, which is attributed to the broad hydrogen-bonded (O-H) group, was observed in both UBC and TBC samples. However, a value of 3268 cm^{-1} was recorded in the OH absorption band of TBC compared to 3280 cm^{-1} in UBC, as shown in Table 4. This suggests that NaOH treatment results in bond fractionation or breaking in the fiber matrix. Furthermore, an absorption band in the range of 1026-1000 cm^{-1} is majorly a characteristic absorption band resulting from C-O stretch in cellulose, hemicellulose and lignin (Akhtar et al. 2016; Bamisaye and Rapheal 2021). A corresponding shift in the C-O stretching with a wave number value of 1013 cm^{-1} observed in TBC compared to 1010 cm^{-1} recorded in UBC could be attributed to delignification due to alkali treatment, as shown in Figure 6.

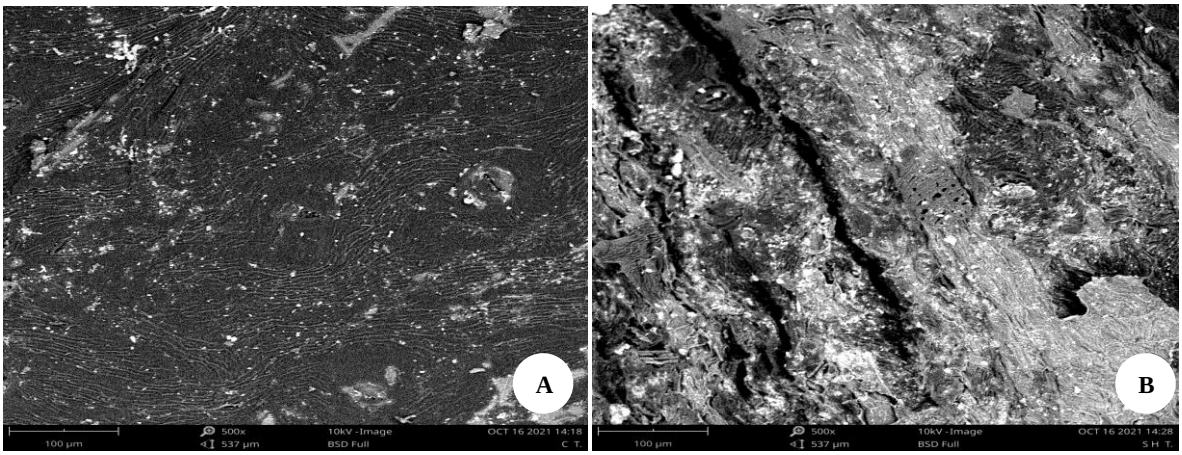


Figure 7. The SEM micrographs of: A. Untreated bean chaff, B. Alkaline Treated bean chaff

The SEM micrograph of untreated bean chaff (UBC) and treated bean chaff (TBC) were presented in Figure 7. The result showed a high degree and clear disruption in the biomass matrix, which could be attributed to the penetration of NaOH in the hemicellulose complex (OH group) in the treated sample, thereby providing a better tackiness and reduction in the lignin content of the biomass compared to 7A in which minimal or no disruption was observed (Bamisaye and Rapheal 2021). However, this corroborates the result obtained in Figure 7B, in which delignification was observed due to the low percentage of lignin contents of the biomass sample of TBC, and also the FTIR result that established that modification, that is, a disruption in the biomass matrix has occurred due to alkali pretreatment compared to the untreated counterpart of the biomass samples.

In conclusion, this study compared the combustion properties of the treated and untreated raw bean chaff and the briquette samples produced with this biomass. The proximate analysis of both the raw chaff and briquettes and the heating value of the bean chaff were determined before and after alkali treatment which improved the quality of the biomass sample. The study's finding reveals that all the essential parameters showed that alkali pretreatment reduced the percentage of Potential Toxic Element (PTE) concentration in the treated sample, as shown by the EDXRF result, thus producing an environmentally friendly briquettes sample. Also, the observed high mean heating value, fixed carbon content, compressive strength, and density of 24.18±0.12 MJ/kg, 30.26±0.01%, 1.11±0.20 N/mm^2 and 0.71±0.10 g/cm^3, respectively of the treated biomass samples compared to the untreated sample was due to the alkali pretreatment process. Furthermore, FTIR analysis was used to confirm bond disruption in the fiber matrices by alkali treatment resulting in delignification at a value of 8±0.01% in TBC with an observed vibrational C-O stretch of 1013 cm^{-1} in the alkali-treated sample (TBC) to 1010 cm^{-1} in UBC. Removing lignin components from the feedstock was necessary because a manually fabricated briquetting machine was adopted. This improved the compactness and porosity, thus showing improved combustion properties of the briquettes sample. In conclusion, alkali-treated briquettes possess better characteristics of a solid biofuel than their untreated counterpart.

ACKNOWLEDGEMENTS

There is no conflict of interest as regards this study. AIA contributed substantially to the conception and design of the research or acquisition of data or analysis and interpretation of data. IAR is involved in drafting the manuscript, acquiring and interpreting data. BA has been involved in drafting the manuscript and revising it critically for important intellectual content. ECM was involved in revising the manuscript. OOA made the general revision of the final manuscript.

REFERENCES

Abayomi B, Rapheal IA, Olaitan BM. 2021. Performance comparison of carbonized and un-carbonized neem leaves briquette. Intl J Adv Acad Res 7 (6): 50-59. DOI: 10.46654/ij.24889849.e767950.

Akhtar MN, Sulong AB, Radzi MF, Ismail NF, Raza MR, Muhamad N, Khan MA. 2016. Influence of alkaline treatment and fiber loading on the physical and mechanical properties of kenaf/polypropylene composites for variety of applications. Prog Nat Sci: Mater Intl 26 (6): 657-664. DOI: 10.1016/j.pnsc.2016.12.004.

Ali MU, Liu G, Yousaf B, Ullah H, Abbas Q, Munir MAMA. 2019. systematic review on global pollution status of particulate matter-associated potential toxic elements and health perspectives in urban environment. Environ Geochem Health 41 (3): 1131-1162. DOI: 10.1007/s10653-018-0203-z.

Amirta R, Nafitri SI, Wulandari R, Yuliansyah, Suwinarti W, Candra KP, Watanabe T. 2016.Comparative characterization of *Macaranga* species collected from secondary forests in East Kalimantan for biorefinery of unutilized fast growing wood. Biodiversitas17: 116-123. DOI: 10.13057/biodiv/d170117.

ASTMS. 2019. American Society for Testing and Materials Standard: Standard Test Methods for Gross Calorific Value of Coal and Coke (ASTM S), D3177-89 (2019).

ASTMS. 2021. American Society for Testing and Materials Standard: Standard Test Methods for Proximate Analysis of Coal and Coke (ASTM S), D3172-13 (2021).

Bamisaye A, Rapheal IA. 2021. Effect of binder type on the NaOH-treated briquettes produced from banana leaves. Biomass Conv Bioref 2021: 1-9. DOI: 10.1007/s13399-021-01771-9.

Bensah EC, Mensah M. 2013. Chemical pretreatment methods for the production of cellulosic ethanol: Technologies and innovations. Intl J Chem Eng 2013: 719607. DOI: 10.1155/2013/719607.

Brand MA, Jacintho RC, Anthunes, R Alexandro BC. 2017. Production of briquettes as a tool to optimize the use of waste from rice cultivation and industrial processing. Renew Energy 111: 116-123. DOI: 10.1016/j.renene.2017.03.084.

Conde-Mejia C, Jimenez-Gutierrez A, El-Halwagi MA. 2012. Comparison of pretreatment methods for bioethanol production from lignocellulosic materials. Process Saf Environ Prot 90 (3): 189-202. DOI: 10.1016/j.psep.2011.08.004.

Deepak KB, Manujesh BJ, Vivek J, Yashas BK. 2019. Development and study of fuel briquettes from areca leaves: A potential renewable energy source. AIP Conf Proc 2080: 03004. DOI: 10.1063/1.5092907.

Demirbas A. 2013. Combustion characteristics of different biomass fuels. Prog Energy Combust Sci 30: 21930. DOI: 10.1016/j.pecs.2003.10.004.

Elinge CM, Oyibo MC, Birnin-Yauri, AU, Ige AR, Yakubu Y, Ogunleye AO. 2020. Enhancing properties of water hyacinth biomass briquettes by mercerization process. Intl Res J Pure Appl Chem 21 (18): 43-55. DOI: 10.9734/irjpac/2020/v21i1830270.

Fernandes ERK, Marangoni C, Souza O, Sellin N. 2013. Thermo-chemical characterization of banana leaves as a potential energy source, Energy Conv Manag 75: 603-608 DOI:10.1016/j.enconman.2013.08.008.

Hordofa GD, Etisa D. 2019. Effect of lime and compost application on the growth and yield of common bean (*Phaseolus Vulgaris* L.): A review. Adv Oceanograph Mar Biol 1 (3): 1-9. DOI: 10.33552/AOMB.2019.01.000512.

Husain Z, Zainac Z, Abdullah Z. 2002. Briquetting of palm fibre and shell from the processing of palm nuts to palm oil. Biomass Bioenergy 22: 505-509. DOI: 10.1016/S0961-9534(02)00022-3.

Ion RM, Zaharia P, Fierascu RC, Ion N. 2007. Analysis of stainless steel samples by Energy Dispersive X-Ray Fluorescence (EDXRF) spectrometry. International Conference on Advanced Technologies and Materials, Galati, Romania.

Jigisha P, Channiwala SA, Ghosal GK. 2007. A correlation for calculating elemental composition from proximate analysis of biomass materials. Fuel 86: 1710-1719. DOI: 10.1016/j.fuel.2006.12.029.

Jyothsna S, Manjula G, Sateesh S, Nageswara AS. 2020. Qualitative elemental analysis of selected potential anti-asthmatic medicinal plant taxa using EDXRF technique. Heliyon 6 (2): e03260. DOI: 10.1016/j.heliyon.2020.e03260.

Karunanithy C, Wang Y, Muthukumarappan K, Pugalendhi S. 2012. Physiochemical characterization of briquettes made from different feedstocks. Biotechnol Res Intl 2012 (2): 165202. DOI: 10.1155/2012/165202.

Krizan P, Soos L, Vukelic DA. 2009. Study of impact technological parameters on the briquetting process. Facta Universitatis Ser: Working Living Environ Prot 6: 39-47.

Lubwama M, Yiga VA. 2017. Development of groundnut shells and bagasse briquettes as sustainable fuel sources for domestic cooking applications in Uganda, Renew Energy 111: 532-542. DOI: 10.1016/j.renene.2017.04.041.

Maciejewska A, Veringa H, Sanders J, Peteve SD. 2006. Co-firing of biomass with coal: constraints and role of biomass pretreatment, Office for Official Publications of the European Communities, Luxembourg

Mahalingam A, Nagappan B, Jayaram P, Nagalingeswara RB. 2019. Investigating the physio-chemical properties of densified biomass pellet fuels from fruit and vegetable market waste. Arab J Sci Eng 45: 563-574. DOI: 10.1007/s13369-019-04294-8.

Mahyati AR, Paton M, Djide N, Taba DP. 2013. Biodegradation of lignin from corn cob by using a mixture of *Phanerochaete chrysosporium*, *Lentinus edodes* and *Pleurotus ostreatus*. Intl J Sci Res 2 (11): 79-82.

Maninder R, Singh K, Grover S. 2012. Using agricultural residues as a biomass briquetting: An alternativesource of energy. J Electr Electr Engi 1 (5): 11-15. DOI: 10.9790/1676-0151115.

Matali S, Rahman NA, Idris SS, Yaacob N, Alias AB. 2016. Lignocellulosic biomass solid fuel properties enhancement via torrefaction. Proced Eng 148: 671-678. DOI: 10.1016/j.proeng.2016.06.550.

Ndiema CKW, Manga PN, Ruttoh CR. 2002. Densification characteristics of rice straw briquettes. J Inst Energy 75 (502): 11-13.

Oyibo MC, Elinge CM, Birnin-Yauri AU, Ige AR, Ogundele OA, Dabai MD. 2020. Combustion profiles of fuel briquettes produced from alkali treated and untreated sugarcane bagasse. Am J Eng Res 9 (3): 268-274.

Rapheal IA, Moki EC, Gusau HL, Abimbola AI, Harrison O. 2018. Effect of binder on physico-chemical properties of fuel briquettes produced from watermelon peels. AASCIT JEnergy 5 (2): 23-27.

Santhebennur JV, Jogttappa N. 2012. Assessment of agro-industrial wastes proximate,ultimate, SEM and FTIR analysis for feasibility of solid bio-fuel production. Univers J Environ Res Technol 2 (6): 575-581.

Wilaipon P. 2007. Physical Characteristics of maize cob briquette under moderate die pressure. Am J Appl Sci 4: 995-998. DOI: 10.3844/ajassp.2007.995.998.

Zhao X, Zhang L, Liu D. 2010. Pretreatment of siam weed stem by several chemical methods for increasing the enzymatic digestibility. Biotechnol J 5 (5): 493-504. DOI: 10.1002/biot.200900284.

Zhao Y, Wang Y, Zhu JY, Ragauskas A, Deng Y. 2008. Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature. Biotechnol Bioeng 99 (6): 1320-1328. DOI: 10.1002/bit.21712.

ASIAN J TROP BIOTECHNOL
Volume 19, Number 1, June 2022
Pages: 28-34

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c190105

Hematological modulatory effects of *Weissella* and *Pediococcus* sp. on formalin-induced inflammation in wistar rats

BABAYEMI OLAWALE OLADEJO♥, HAWAH MORIYIKE OLUWASOLA
Department of Microbiology, Federal University of Technology. P.M.B. 704, Akure, Nigeria.
Tel./fax.: +234-9042422526, ♥email: booladejo@futa.edu.ng

Manuscript received: 6 June 2022. Revision accepted: 29 June 2022.

Abstract. *Oladejo BO, Oluwasola HM. 2022. Hematological modulatory effects of* Weissella and Pediococcus *sp. on formalin-induced inflammation in wistar rats. Asian J Trop Biotechnol 19: 28-34.* The activity of three LAB strains, *Weissella cibaria* II-1-59, *Weissella confusa* JMC 1093, and *Pediococcus pentosaceus* DSM20336, isolated from a Nigerian locally fermented food condiment; 'iru' on blood parameters of acutely inflamed rats using formalin (1%) was investigated in this study. The rats were distributed into six groups (A-F). Rats in Groups A were neither administered formalin nor treated with LAB, while Group B received formalin injection only. Groups C-E were treated with the strains of LABs (2 × 107 CFU/mL), while Group F received diclofenac sodium treatment (150 mg/kg body weight) following formalin administration. In addition, Erythrocyte sedimentation rate **(**ESR), total and differential white blood cell (WBC) count, and total red blood cell (RBC) count were analyzed using standard methods. LAB Treatments significantly reduced the ESR of the blood of LAB-treated rats (1.00±0.00 mm/hr at 1 hour) at $P<0.05$ and regulated leucocyte infiltration in the blood circulation (*W. cibaria* treated group with a neutrophil count of 13.67±1.20% at 336 hrs) compared to diclofenac sodium. These findings revealed that the LAB were good immune modulators and would be effective agents for the treatment of inflammation-induced anemia.

Keywords: Formalin, hematology, inflammation, *Lactobacillus*, edema

INTRODUCTION

Inflammation is a routine immune response used to subdue infectious agents or infections that get into the body tissues (Paramita et al. 2017; Szalay 2018). This inflammatory reaction usually facilitates healing processes, however, it may become fatal if uncontrolled (Sung-Min et al. 2019). Anemia of inflammation (AI), also referred to as anemia of chronic disease or anemia of chronic disorders, is occasionally characterized by a mild to moderately severe anemia that arises during infection, such as in the case of inflammatory disease and or malignancy (Nemeth and Ganz 2014). Unfortunately, there seem to be less efficient laboratory measures required to assess patients suffering from chronic inflammation. Also, diagnoses are only initiated when the inflammation occurs alongside other medical conditions, as observed in the case of cytokine storms linked with the severity of COVID-19 infection.

The development of AI within the first few hours after the commencement of inflammation is sustained by the activation of immune cells and the release of pro-inflammatory chemical mediators such as interferon interferon-γ, interleukin-1, interleukin 9, and tumor necrosis factor-α (TNF-α) (Nairz et al. 2016). As such, alleviating these pro-inflammatory cytokines is highly important for improving inflammatory disorders (Oladejo and Oluwasola 2021). Also, a mild reduction in erythrocyte survival combined with the weakened production of erythrocytes has been linked with AI disorder. This increased destruction of the erythrocytes is mainly associated with the activation of macrophages by inflammatory cytokines; however, other hemolytic mechanisms may play a significant role in specific inflammatory disease conditions. Direct cytokine reaction on the erythropoietic precursors and iron restriction associated with the suppression of erythrocyte production. This can lead to a reduction in the numbers but usually of normal hemoglobin content and size (normocytic, normochromic anemia) (Nemeth and Ganz 2014).

Anemia of inflammation can, therefore, significantly contribute to the morbidity of arrays of inflammatory disorders or diseases occasionally in patients. Treatment options include blood transfusion (although risk associated) as well as the use of iron supplements and erythropoiesis-stimulating agents (ESAs) via intravenous administration (Steinbicker et al. 2011). Although some acceptable outcomes have been achieved with the use of conventional drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and herbal supplements in the treatment of inflammatory disorders, a life-threatening condition of chronic cases which poses a major threat to the health status and longevity of individuals persist (Barcelos et al. 2019). As such, there is the need to explore more naturally available alternative therapy for AI., such as in the case of lactic acid bacteria, which are generally regarded as safe (GRAS).

Fermented locust bean ('Iru') is a popular food condiment consumed in Nigeria and other West African countries. It is a very good source of many beneficial microbial strains, particularly the genera of *Bacillus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. Also, it is

often consumed as a good alternative to meat due to its high protein, fat, tannin, vitamins, and mineral contents. These organisms form part of major probiotics, which are live organisms that confer significant health benefits when administered in sufficient amounts in the host (Afolabi et al. 2016). Lactic acid bacteria (LAB) are also known as probiotics and can serve as immune modulators (Pessione 2012). They have been isolated from various fermented foods and used in the production of various fermented foods and beverages. A previous study by Oladejo and Oluwasola (2021) showed that *Weissella* and *Pediococcus* sp. have anti-inflammatory potentials and suppressed the effect of acute inflammation in rats via cytokine regulations (interleukin-10, C-reactive protein, and transforming growth factor-β). Formalin is a strong chemical that has been predominantly explored in the induction of inflammation. Formalin-induced paw edema in a rat is, therefore, one of the most suitable tests to screen for inflammatory responses. Therefore, this study aims to assess the effect of oral administration of lactic acid bacteria isolated from indigenous fermented locust beans ('Iru') on the hematological parameters of Wistar rats induced with formalin.

MATERIALS AND METHODS

Collection of fermented locust bean ('Iru')

Fermented locust bean ('Iru') was purchased from Oba market in Akure, Ondo State (7°10'N 5°05'E), Nigeria. They were put in a sterile polythene bag and transported immediately to the Department of Microbiology Laboratory, Federal University of Technology, Akure, Nigeria, for bacteriological analysis.

Isolation and identification of lactic acid bacteria (LAB)

Lactic acid bacteria from the fermented locust bean ('Iru'), including *Weissella cibaria* II-1-59, *Weissella confusa* JMC 1093, and *Pediococcus pentosaceus* DSM20336 were isolated and identified as described in the previous work of Oladejo and Oluwasola (2021). Briefly, molecular identification of the isolated LAB was carried out by harvesting cell pellets from 2 mL of overnight cultures (up to 2×10^9 cells) of LAB grown in MRS broth and DNA extraction was done using a JenaBioscience DNA Purification Kit following the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out to amplify the 16SrRNA gene of the bacteria using the primer pair 27F-5′-AGAGTTTGATCCTGGCTCAG-3', and 1492R 5'GGTTACCTTGTTACGACTT-3'. The amplified product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80 V for 1 hour 30 minutes. Following electrophoresis, DNA bands were visualized by ethidium bromide staining with 100 bp DNA ladder (Solis Biodyne) used as DNA molecular weight marker (Oladejo and Oluwasola 2021). They were BLAST-searched to detect similar sequences in the NCBI database (https://www.ncbi.nlm.nih.gov).

Inflammatory drugs and chemicals

Formalin (Pascal Biosciences) and diclofenac sodium drug (Impulse Pharma Pvt. Ltd, Boisar, India. Expiry date; March, 2023) were purchased in Akure, Nigeria.

Animal and experimental design

Eighty-four (84) male Wistar rats weighing 120 -150 kg used in this research were obtained from the Department of Microbiology, Federal University of Technology, Akure (FUTA), Nigeria. They were housed in cages with wire screen tops and maintained under adequate ventilation and environmental temperature. The animals were maintained on a commercial rat chow with tap water, food (finisher) provided and acclimatized for one week before the experimental session. All the experimental procedures were carried out following the guidelines of the Institutional Animals Ethics Committee of the Federal University of Technology Akure, Nigeria. Ethical permission was sought from FUTA Research Ethical Committee (FUTA/ETH/21/06).

Evaluation of anti-inflammatory activity

Inflammation was induced by sub-plantar injection of 0.1 mL of 1% freshly prepared formalin into the right hind paws of all the rat groups except the Group A rats (formalin control). After inflammation had been induced, the rats were divided into 5 groups (Groups B-F) of 14 rats each. Rats in Group B were not treated following inflammation (negative control); rats in Group C, D, and E were treated orally with 2×10^7 CFU/mL of *W. cibaria* II-1-59, *W. confusa* JMC 1093, and *P. pentosaceus* DSM20336 respectively while rats in Group F (positive control) were treated with diclofenac sodium (150 mg/Kg body weight) after the development of paw edema in all the rats. Changes in rat paw thickness were measured at 20 min before and after injection at different time intervals (0, 1, 4, 8, 24, 72, 168 and 336 h) using a digital vernier caliper and were measured in millimeters (mm) (Amdekar et al. 2012).

Blood sample collection

At earlier intervals, the rats were sacrificed through cardiovascular bleeding according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). Then, blood samples were collected into an EDTA bottle for hematological assays. The hematological parameters such as erythrocyte sedimentation rate **(**ESR), total and differential white blood cell count, and total red blood cell count were analyzed using the method of Valentini et al. (2015) as stated below.

Determination of erythrocyte sedimentation rate (ESR)

Erythrocyte Sedimentation Rate (ESR) was estimated by adding dilution of the blood sample with sodium citrate anticoagulant into a Westergren tube until the blood level reached 100 millimeters (mm). The tubes were stored vertically and allowed to sit at room temperature for an hour. The distance between the top of the blood mixture and the top of the sedimentation of RBCs was measured. The ESR (mm) was calculated using the formula: ESR (mm) = Final reading – initial reading.

Determination of white blood cell count (WBC)

The blood was first diluted in ratio 1: 20 and 0.02 mL of the blood was pipetted into 0.38 mL of diluting fluid, Turk's reagent (3 % acetic acid with crystal violet dye). Then, a small portion was charged into the counting chamber and observed using x40 objective to count the cell in cells/cubic mm. Finally, the total number of WBCs counted was calculated using the formula:

WBCs = number of cells counted x depth factor (10) x dilution factor x area factor (0.25).

Determination of red blood cell count (RBC)

The blood sample was diluted 1: 200 and mixed properly. About 0.02 mL of the blood was pipetted into 4 mL of diluting fluid in a bijou bottle and was mixed thoroughly by alternately drawing up expelling fluid. A Pasteur pipette was used to fill the counting chamber and red blood cells were counted under x40 objective. The total number of RBCs counted was calculated using the formula:

RBCs = number of cells counted x dilution factor (200) x 1/5 x area factor (0.1 mm^3).

Determination of leucocyte differential count

A drop of blood was thinly spread over a glass slide and air dried. The smear was covered with Leishman stain and allowed to stand for 2 minutes after which it was buffered with distilled water and allowed to stand for 10 minutes. The slide was then rinsed thoroughly with distilled water, air dried and viewed under a microscope. Different WBC (neutrophil, eosinophil, lymphocyte and monocyte) were counted and numbers recorded.

Statistical analysis

Data were expressed as the mean ± standard error mean (SEM) calculated over an independent time frame of experiments performed in duplicate. One-way analysis of variance (ANOVA) was applied, followed by a post hoc test; Bonferroni's Multiple Comparison Test for difference between treatments groups compared with control (Group B) using GraphPad Prism version 5.0.

RESULTS AND DISCUSSION

Effects of oral treatment of LAB on erythrocyte sedimentation rate (ESR)

The sub-plantar injection of formalin into the right hind paw of the rats caused an increase in the erythrocyte sedimentation rate (ESR) of the injected groups reaching the maximum rate at 1 hour and then reducing continuously till the end of the experiment, as shown in Figure 1. In Group A rats (formalin control), the ESR was 0.30±0.00 mm/hr at 1 hour, which remained the same throughout the period of the experiment. The highest erythrocyte sedimentation rate (4.00±0.00 mm/hr) was observed in group B and C rats 1 hour after the injection, which was significant at $p<0.05$. Group E and F rats showed the lowest ESR of 1.00±0.00 mm/hr at 1 hour. A significant decrease was immediately observed in every group except Group A after 1 hour and continued till the end of 336 hours.

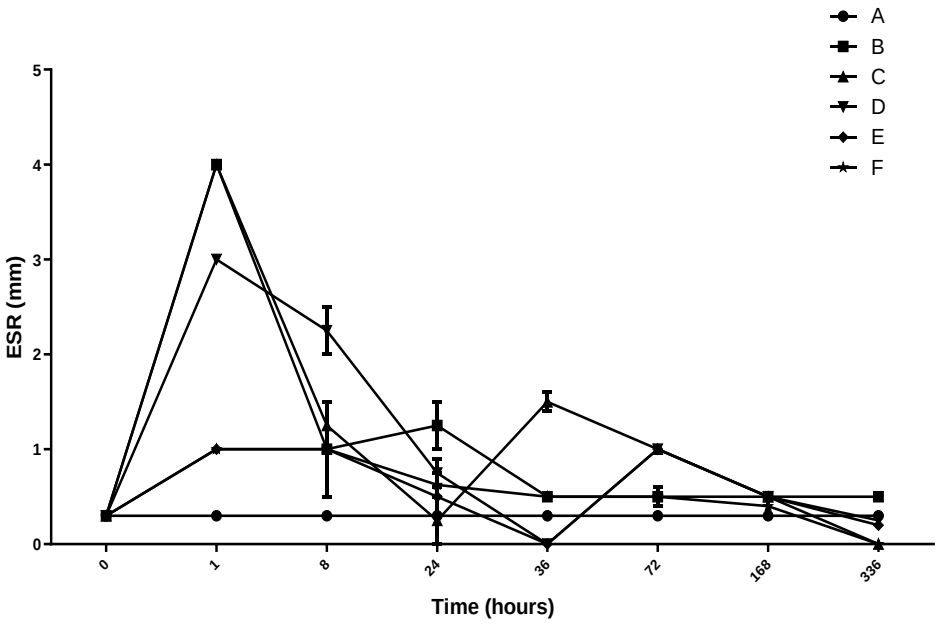


Figure 1. Effect of oral treatment of LAB on erythrocyte sedimentation rate [ESR] (mm). Note: n =2 (significant at P<0.05). Data are expressed as mean ± standard error of two rats per group. Group A: formalin control; Group B: formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control)

Effects of oral treatment of LAB on the total white blood cell count of rats

In Group A (formalin control group), total white blood cell count at 1 hour was $4.68\pm0.00 \times 10^3$ μL^{-1} which remained the same throughout the period of the experiment. The WBC count of Group B rats at 1 hour was $3.40\pm0.38 \times 10^3$ μL^{-1} which increased gradually till the end of the experiment. LAB treated Groups C, D and E rats showed WBC counts of $2.85\pm0.00 \times 10^3$ μL^{-1}, $5.55\pm0.00 \times 10^3$ μL^{-1} and $3.95\pm1.60 \times 10^3$ μL^{-1}, respectively, at 1 hour which increased up to the end of 36 hrs, followed by a decrease at 72 hours till 336 hours. Group F rats (Diclofenac treated group) showed the most decrease in the WBC count at 1 hour to be $2.43\pm0.75 \times 10^3$ μL^{-1} as shown in Table 1.

Effects of oral treatment of LAB on the total red blood cell count of rats

Total red blood cell count of Group A rats (formalin control) at 1 hour was $8.65\pm0.41 \times 10^6$ μL^{-1} which remained constant throughout the period of the experiment. In untreated Group B rats, a continuous low RBC count was observed until after 168 hrs. LAB treated Groups C, D and E rats showed RBC counts of $6.00\pm0.03 \times 10^6$ μL^{-1}, $4.83\pm0.12 \times 10^6$ μL^{-1} and $4.33\pm0.06 \times 10^6$ μL^{-1}, respectively, within the first hour of the experiment. An increase in RBC count was observed at 8 hours and then continued till the end of the experiment. Relatively low RBC count was observed in Group F (Diclofenac treated group) until the end of 72 hours compared with other treatment groups, as shown in Table 2.

Effects of LAB treatment on the percentage of circulating lymphocytes in rats

The percentage circulating lymphocyte count of 80.50±2.02% was observed in Group A rats (formalin control) at 1 hour, which remained constant throughout the period of the experiment. After 1 hour, a significant reduction in the population of circulating lymphocytes was observed in all other groups. In untreated Group B rats, there was a continuous reduction in the percentage of circulating lymphocyte count till the end of 72 hours. But in Group C, D, E, and F, a significant increase was observed after 24 hours and continued till the end of the experiment. The percentage of circulating lymphocyte count observed in Group F (Diclofenac treated group) at 1 hour was lower compared with other treatment groups, as shown in Table 3.

Table 1. Effects of LAB treatment on the total white blood cell count (WBC) x 10^3 μL^{-1}

Groups	Time (hrs)						
	1	8	24	36	72	168	336
A	4.68±0.00**	4.68±0.00**	4.68±0.00**	4.68±0.00**	4.68±0.00**	4.68±0.00**	4.68±0.00**
B	3.40±0.38	3.55±2.08	4.00±0.18	5.05±0.25	5.25±0.38	5.40±0.15	5.50±0.05
C	2.85±0.00**	5.50±0.00**	9.05±0.03	11.40±0.00**	9.10±0.00**	8.55±0.00**	4.85±1.83**
D	5.55±0.00	5.40±0.00**	10.07±0.60**	11.95±0.05**	9.45±0.00**	4.75±0.00**	4.15±1.73**
E	3.95±1.60	6.45±0.00	8.20±1.68	8.65±0.67	8.55±0.00	7.60±0.00	5.15±0.00
F	2.43±0.75**	3.85±0.00	6.20±0.90	9.55±0.00	3.52±1.17	2.50±0.00	5.00±0.00

Note: Data are expressed as mean ± standard error of two rats per group. Group A: formalin control; Group B: formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with P. pentosaceus DSM20336; Group F: treatment with diclofenac sodium (positive control). **Difference between comparison treatment group was considered statistically significant at $P < 0.05$ when compared with control group (B)

Table 2. Effects of LAB treatment on the total red blood cell count (RBC) x 10^6 μL^{-1}

Groups	Time (hrs.)						
	1	8	24	36	72	168	336
A	8.65±0.41**	8.65±0.41**	8.65±0.41**	8.65±0.41**	8.65±0.41**	8.65±0.41**	8.65±0.41**
B	4.15±0.43	4.48±0.50	4.30±0.65	5.23±0.50	5.69±0.42	5.50±0.75	8.05±0.54
C	6.00±0.32**	6.10±0.03**	6.82±0.08**	7.45±0.31**	6.09±0.11	7.40±0.20**	8.24±1.10
D	4.83±0.12	6.05±0.34**	6.15±0.33**	7.25±0.28**	7.55±0.06	7.73±0.36**	7.91±0.28
E	4.33±0.06	6.52±0.66**	7.08±0.90**	7.21±0.15**	7.86±1.33**	7.48±0.26**	8.25±0.27
F	3.81±0.75	4.71±0.13	5.65±0.18	5.74±0.52	6.64±0.18	9.72±1.15	9.11±1.08

Note: Data are expressed as mean ± standard error of two rats per group. Group A: formalin control; Group B: formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). **Difference between comparison treatment group was considered statistically significant at $P < 0.05$ when compared with control group (B)

Table 3. Effects of LAB treatment on total percentage circulating lymphocyte count (%)

Groups	Time (hrs.)						
	1	8	24	36	72	168	336
A	80.50±2.02	80.50±2.02**	80.50±2.02**	80.50±2.02**	80.50±2.02	80.50±2.02	80.50±2.02
B	76.00±2.08	61.33±0.88	61.67±2.73	64.67±1.20	73.33±1.45	75.33±2.40	81.67±0.88
C	71.00±1.00	68.00±1.15	58.67±2.91	75.67±1.20**	78.67±1.20	80.00±1.15	83.33±1.45
D	72.00±1.73	70.67±2.33**	64.00±1.73	76.66±1.33**	75.33±2.40	78.00±1.53	79.67±3.28
E	69.34±1.33	67.00±1.53	63.00±1.15**	74.67±0.33**	78.33±1.20	82.00±1.73	85.33±1.33
F	62.00±1.53	60.00±0.58	69.00±1.15	68.00±1.53**	71.00±0.57	80.34±1.45	81.00±0.58

Note: Data are expressed as mean ± standard error of two rats per group. Group A: formalin control; Group B: formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). **Difference between comparison treatment group was considered statistically significant at P <0.05 when compared with control group (B)

Effects of LAB treatment on the percentage of circulating neutrophils in rats

Sub-plantar injection of formalin into the rat paw caused infiltration of neutrophils in blood circulation, which led to an increase in the percentage circulating neutrophil count in all the injected Groups within 8 hours of the experiment. In Group A rats (formalin control), the total percentage neutrophil count was 17.50±1.44% at 1 hour, which remained the same throughout the period of the experiment. The highest percentage circulating neutrophil count (38.33±4.33%) was observed in Group B rats at 8 hours, while the lowest was observed in Group C rats (13.67±1.20%) at 336 hours. In LAB-treated Groups C, D, and E rats, a significant decrease in percentage circulating neutrophil count was observed after 24 hours and then continued till the end of the experiment. The percentage neutrophil count observed in Group F (Diclofenac treated group) was higher when compared with other treatment groups, as shown in Table 4.

Effects of LAB treatment on the percentage of circulating monocyte in rats

In Group A rats (formalin control), the percentage circulating monocyte count was 1.67±0.33% at 1 hour, which remained the same throughout the period of the experiment. A significant increase in the population of circulating monocyte was observed in all other groups after 1 hour. The highest percentage of circulating monocyte was observed in Group B rats (3.82±0.00%) at 8 hours, while the lowest was observed in Groups C and E rats (0.67±0.33%) at 336 hours. In LAB treated groups, a significant reduction in the population of circulating monocyte was observed after 8 hours which continued till the end of 336 hours, as shown in Table 5.

Table 4. Effects of LAB treatment on total percentage circulating neutrophil count (%)

Groups	Time (hrs)						
	1	8	24	36	72	168	336
A	17.50±1.44**	17.50±1.44**	17.50±1.44**	17.50±1.44**	17.50±1.44**	17.50±1.44**	17.50±1.44
B	24.33±2.33	38.33±4.33	35.00±2.89	32.33±1.20	22.67±1.20	24.67±0.88	16.00±1.00
C	25.67±0.33	31.66±1.20	26.67±1.76**	22.00±1.15**	19.00±0.58	16.67±0.88**	13.67±1.20
D	27.00±1.15	32.33±2.67**	29.67±3.84**	19.00±1.15**	22.00±3.51	17.67±0.88	15.67±2.85
E	27.33±1.20	32.33±2.02	24.00±1.16**	23.33±1.45**	18.67±1.76	15.33±1.76**	14.67±1.45
F	36.33±1.67**	33.00±0.58	36.00±1.16	27.00±2.08**	23.00±1.73	18.67±1.45	18.00±0.58

Note: Data are expressed as mean ± standard error of two rats per group. Group A: formalin control; Group B: formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). **Difference between comparison treatment group was considered statistically significant at P <0.05 when compared with control group (B)

Table 5. Effects of LAB treatment on total percentage circulating monocyte count (%)

Groups	Time (hrs)						
	1	8	24	36	72	168	336
A	1.67±0.33	1.67±0.33	1.67±0.33	1.67±0.33	1.67±0.33	1.67±0.33	1.67±0.33
B	1.00±0.33	3.82±0.00	3.00±0.33	2.00±0.58	2.33±0.66	1.67±0.33	1.33±0.33
C	1.67±0.33	3.33±0.33**	2.66±0.33	2.33±058	2.00±0.33	1.33±0.58	0.67±0.00**
D	2.33±0.33	3.67±0.33**	3.34±0.33**	2.67±0.33**	2.33±0.33	1.67±0.33	1.70±0.00
E	2.34±0.33**	2.67±0.33	2.33±0.33	2.00±0.58	1.33±0.33	1.00±0.33	0.67±0.00**
F	1.67±0.33	2.00±0.58	3.00±0.58	2.33±0.33	0.33±0.00**	1.33±0.00	1.00±0.33

Note: Data are expressed as mean ± standard error of two rats per group. Group A: formalin control; Group B: formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). **Difference between comparison treatment group was considered statistically significant at P <0.05 when compared with control group (B)

Discussion

Lactic acid bacteria isolated from African fermented locust beans (Iru) in this study appear similar to other studies that have successfully isolated and identified different types of LAB from fermented food products, including African locust beans (Uaboi-Egbenni et al. 2009; Ouoba et al. 2010; Ajayi 2014; Afolabi et al. 2016; Oyetola et al. 2017). Therefore, they are regarded as a major group of probiotic bacteria with a sufficient number of studies revealing their health-promoting ability in man and animals.

Blood cells play a major significant role in the onset of inflammation. The aggregation of white blood cells (Leucocytes) at the site of inflammation is a key elemental event in the inflammatory process, where adhesion and cell mobility processes could lead to cell migration (Umapathy et al. 2010). Treatment with *W. cibaria* II-1-59; *W. confusa* JMC 1093, and *P. pentosaceus* DSM20336 significantly decrease the WBC count, which suggests that these LAB strains can regulate the migration of WBCs to the site of inflammation which might have led to their significant decrease in the paw thickness of LAB treated Groups C, D and E rats. However, it was observed that *W. cibaria* II-1-59 and *W. confusa* JMC 1093 had a better ability to regulate this leucocyte infiltration in the inflamed rats' blood circulation than *P. pentosaceus* DSM20336. Moreover, studies by Szabo et al. (2011) and Tsai et al. (2014) further supported that treatment with LAB strains had a statistical significance on the hematological parameters in rats.

Red blood cells (Erythrocytes) and hemoglobin are also important for oxygen transportation. It is well known that inflammation can cause a significant decrease in the RBC, leading to erythropenia (Straat et al. 2012). LAB strains in this study showed a better ability to regulate the decrease in the RBC of the inflamed rats. An increase in RBC count was observed in LAB-treated rats compared to diclofenac sodium. This result was in line with the study of Bikheet et al. (2021), which showed that oral administration of LAB (*P. acidilactaci*) caused a significant increase in the RBC count of treated rats. A high level of RBC shows that the rats were not anemic, while a lower level indicates a sign of anemia (Cheesborough 1991). LABs may have used mechanisms to disrupt RBC-endothelial interactions, thereby modulating the rats' excessive development of anemic conditions as described by several researchers (Adeboboye et al. 2022). They might have also been able to avoid prolonged erythropenia by controlling the release of acute phase proteins like fibrinogen, as shown in the ESR results of this study.

Erythrocyte sedimentation rate (ESR) measures the rate at which RBCs settle in anticoagulated blood, which is a process influenced by the presence of fibrinogen, an acute phase protein associated with inflammatory response (Bray et al. 2016). An elevated ESR due to an increase in the level of fibrinogen serving as a significant mediator of RBC aggregation, therefore, indicates the existence of inflammation. The sub-plantar injection of formalin into the right hind paws of the rats in this study induced an inflammatory response within the first hour based on the swelling of the rat paw and an increase in the ESR. However, a significant ESR decrease ($P<0.05$) in the blood of LAB-treated rats indicated that *W. cibaria* II-1-59; *W. confusa* JMC 1093, and *P. pentosaceus* DSM20336 were able to control the release of fibrinogen in the blood thereby alleviating inflammatory response.

The reduction in the population of neutrophils and monocytes after the treatment with these LAB strains showed their involvement in suppressing the inflammatory response. The *W. cibaria* II-1-59 had the best ability to control the influx of neutrophils, which is essential for sustaining inflammatory response and the release of pro-inflammatory cytokines. The control of leucocyte infiltration could result from the production of specific cytokines responsible for regulating the responses of arrays of immune cells (Scheiermann et al. 2015). Also, the lymphocyte counts of LAB-treated rates were higher compared to diclofenac-treated rats (positive control group). The primary role of lymphocytes is associated with antibody formation (humoral) and cellular immunity. Therefore, a marked increase in the lymphocyte count observed in LAB-treated rats showed signs of an immunostimulatory effect. In a previous study, Aboderin and Oyetayo (2006) reported that the lactic acid bacterium (*Lactobacillus plantarum*) isolated from fermenting corn slurry has immunostimulatory properties due to raised lymphocyte count in rats. Also, Aattouri et al. (2002) had also earlier reported that oral ingestion of lactic acid bacteria increases lymphocyte proliferation and interferon-γ production.

These immune cell responses can be due to findings of our previous study, Oladejo and Oluwasola (2021), which reported that the oral administration of *Weissella* and *Pediococcus* sp. significantly decreased inflammatory responses in rats. This showed that treatment with these LAB had a very consistent anti-inflammatory effect and thus compared favorably with diclofenac sodium (standard NSAID drug) through the ability to modulate inflammatory responses via control of the balance between the pro- and anti-inflammatory cytokines (Groeger et al. 2013), as our treatments with *Weissella* and *Pediococcus* sp. significantly decreased the secretion of pro-inflammatory biomarker (CRP) and increased the serum IL-10 and TGF-β concentration of LAB treated rats (Oladejo and Oluwasola 2021).

In conclusion, the results of oral administration of W. cibaria II-1-59; *W. confusa* JMC 1093, and *P. pentosaceus* DSM20336 on the hematological parameters in the rats showed that these LAB strains could ameliorate inflammatory-induced anemia. Therefore, treating anemia of inflammation with lactic acid bacteria would be a great breakthrough, considering the risks associated with the treatment, as mentioned earlier.

REFERENCES

Aattouri N, Bouras M, Tome D, Marcos A, Lemonnier D. 2002. Oral ingestion of lactic acid bacteria by rats increases lymphocyte proliferation and interferon-γ production. Br J Nutr 87 (4): 367-373. DOI: 10.1079/bjnbjn2001527.

Aboderin FI, Oyetayo VO. 2006. Hematological studies of rats fed different doses of probiotic, *Lactobacillus plantarum*, isolated from fermenting corn slurry. Pak J Nutr 5 (2): 102-105. DOI: 10.3923/pjn.2006.102.105.

Adeboboye CF, Oladejo BO, Adebolu TT. 2022. Effects of *Lactobacillus fermentum* on inflammatory-associated anaemia and leucocyte homeostasis in carrageenan-induced paw edema in wistar rats. Bact Emp 5 (1): e327. DOI: 10.36547/be.327.

Afolabi FT, Abdulkadir M, Onilude A. 2016. Isolation and screening of microorganisms associated with locust bean (Iru) for the ability to ferment soya bean to produce soy Iru. Br Microbiol Res J 13 (6): 1-10. DOI: 10.9734/BMRJ/2016/24593.

Ajayi AO. 2014. Bacteriology and Qualitative study of African locust bean (*Parkia biglobosa*). Open J Soc Sci 2: 73-78. DOI: 10.4236/jss.2014.211010.

Amdekar S, Roy P, Singh V, Kumar A, Singh R, Sharma P. 2012. Anti-inflammatory activity of *lactobacillus* on carrageenan-induced paw edema in male wistar rats. Intl J Inflam 75: 1-6. DOI: 10.1155/2012/752015.

Barcelos IP Troxel RM, Graves JS. 2019. Mitochondrial dysfunction and multiple sclerosis. Biology 8 (2): 37. DOI: 10.3390/biology8020037.

Bikheet MM, Mahmoud ME, Hassan HM, Yassien EE. 2021. The effect of two strains of LAB (*P. acidilactaci* and *L. lactis*) in laboratory rats. SJAS 3 (1): 137-143. DOI: 10.21608/sjas.2021.73876.1093.

Bray C, Bell LN, Liang H, Haykal R, Kaiksow F, Mazza JJ, Yale SH. 2016. Erythrocyte sedimentation rate and c-reactive protein measurements and their relevance in clinical medicine. WMJ 115 (6): 317-321.

Cheesborough M. 1991. Medical Laboratory Manual for Tropical Countries. 2nd Edition. Tropical Health Technology and Butterworth Scientific Limited, London.

Groeger DO, Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, Shanahan F, Quigley EM. 2013. *Bifidobacterium infantis* 35624 modulates inflammatory processes beyond the gut. Gut Microbes 4 (4): 325-339. DOI: 10.4161/gmic.25487.

Nairz M, Theurl I, Wolf D, Weiss G. 2016. Iron deficiency or anemia of inflammation? Differential diagnosis and mechanisms of anemia of inflammation. Wien Med Wochenschr 166: 411-423. DOI: 10.1007/s10354-016-0505-7.

Nemeth E, Ganz T. 2014. Anemia of inflammation. Hematol Oncol Clin North Am 28 (4): 671-681. DOI: 10.1016/j.hoc.2014.04.005.

Oladejo BO, Oluwasola HM. 2021. Oral administration of *Weissella* and *Pediococcus* sp. alleviates formalin induced inflammation in rats by cytokine modulation. Ife J Sci 23 (2): 1-10. DOI: 10.4314/ijs.v23i2.10.

Ouoba LI, Nyanga-Koumou CA, Parkouda C, Sawadogo H, Kobawila SC, Keleke S, Diawara B, Louembe D, Sutherland JP. 2010. Genotypic diversity of lactic acid bacteria isolated from African traditional alkaline-fermented foods. J Appl Microbiol 108: 2019-2029. DOI: 10.1111/j.1365-2672.2009.04603.x.

Oyetola MT, Agarry O, Oyetola S. 2017. Screening of indigenous bacterial isolates from dawadawa (African locust beans) in North Central Nigeria. Biochem Mol Biol 2(6): 67-72. DOI: 10.11648/j.bmb.20170206.11.

Paramita S, Kosala K, Dzulkifli D, Saputri DI, Wijayanti E. 2017. Anti-inflammatory activities of ethnomedicinal plants from Dayak Abai in North Kalimantan, Indonesia. Biodiversitas 18: 1556-1561. DOI: 10.13057/biodiv/d180434.

Pessione E. 2012. Lactic acid bacteria contribution to gut microbiota complexity: Lights and shadows. Front Cell Infect Microbiol 2 (86): 1-15. DOI: 10.3389/fcimb.2012.00086.

Scheiermann P, Frenette PS, Hidalgo A. 2015. Regulation of leucocyte homeostasis in the circulation. Cardiovasc Res 107 (3): 340-351. DOI: 10.1093/cvr/cvv099.

Steinbicker AU, Sachidanandan C, Vonner AJ, Yusuf RZ, Deng DY, Lai CS, Rauwerdink KM, Winn JC, Saez B, Cook CM, Szekely BA, Roy CN, Seehra JS, Cuny GD, Scadden DT, Peterson RT, Bloch KD, Yu PB. 2011. Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. Blood 117 (18): 4915-4923. DOI: 10.1182/blood-2010-10-313064.

Straat M, van Bruggen R, de Korte D, Juffermans NP. 2012. Red blood cell clearance in inflammation. Transfus Med Hemother 39: 353-360. DOI: 10.1159/000342229.

Sung-Min H, Gehoon C, Yong HK, Chul-Kyu P. 2019. The role of maresins in inflammatory pain: Function of macrophages in wound regeneration. Intl J Mol Sci 20 (5849): 1-16. DOI: 10.3390/ijms20235849.

Szabo NJ, Laurie C, Dolana GA, Burdocka TS, Satob HS, Tohru U, Satoko Y, Masamichi T, Hirofumi U. 2011. Safety evaluation of *Lactobacillus pentosus* strain b240. Food Chem Toxicol 49 (1): 251-258. DOI: 10.1016/j.fct.2010.10.027.

Szalay J. 2018. Inflammation: Causes, Symptoms and Anti-inflammatory Diet. www.livescience.com Accessed 07/04/2020.

Tsai YT, Cheng PC, Pan TM. 2014. Anti-obesity effects of gut microbiota are associated with lactic acid bacteria. Appl Microbiol Biotechnol 98: 1-10. DOI: 10.1007/s00253-013-5346-3.

Uaboi-Egbenni PO, Okolie PN, Sobande AO, Alao O, Teniola O, Bessong PO. 2009. Identification of subdominant lactic acid bacteria in dawadawa (a soup condiment) and their evolution during laboratory-scale fermentation of *Parkia biglobosa* (African locust beans). Afr J Biotechnol 8 (25): 7241-7248. DOI: 10.5897/AJB2009.000-9558.

Umapathy E, Ndebia EJ, Meeme A, Adam B, Menziwa P, Nkeh-Chungag BN, Iputo JE. 2010. An experimental evaluation of *Albuca setosa* aqueous extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation. J Med Plant Res 4 (9): 789-795. DOI: 10.5897/JMPR10.056.

Valentini L, Pinto A, Bourdel-Marchasson I, Ostan R, Brigidi P, Turroni S. 2015. Impact of personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota - The "RISTOMED project": Randomized controlled trial in healthy older people. J Clin Nutr 34: 593-602. DOI: 10.1016/j.clnu.2014.09.023.