Cell Biology & Development

| Cell Biol Dev | vol. 7 | no. 1 | June 2023 | | E-ISSN 2580-4499|

Allium fistulosum L. photo by Forest and Kim Starr

Cell Biology & Development

| Cell Biol Dev | vol. 7 | no. 1 | June 2023 | E-ISSN 2580-4499|

Published semiannually **PRINTED IN INDONESIA**

9 772580 282138

Cell Biology & Development

| Cell Biol Dev | vol. 7 | no. 1 | June 2023 |

ONLINE
http://smujo.id/cbd

e-ISSN
2580-4499

PUBLISHER
Society for Indonesian Biodiversity

CO-PUBLISHER
Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia

OFFICE ADDRESS
Indonesian Legumes and Tuber Crops Research Institute. Jl. Raya Kendalpayak Km 8, Po. Box 66, Malang 65101, East Java, Indonesia. Tel.: +62-341-801468, Fax.: +62-341-801496, email: editors@smujo.id

PERIOD OF ISSUANCE
June, December

Society for Indonesian Biodiversity

Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia

Published by Smujo International for The Society for Indonesian Biodiversity and Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia

GUIDANCE FOR AUTHORS

Aims and Scope ***Cell Biology and Development*** **(Cell Biol Dev)** encourages submission of manuscripts dealing with all aspects of the cells biology, plant molecular biology and biotechnology including organelles and cellular compartments, trafficking and turnover, signaling, motility, adhesion, cell division, differentiation and programmed cell death, regeneration, organogenesis and somatic embryogenesis, gene transfer, gene flow, secondary metabolites, metabolic engineering, impact of transgene(s), physiological, pharmacological, and toxic response of cellular systems; genomics and genetics, metabolism, abiotic and biotic stress, phytopathology, gene transfer and expression, molecular pharming, systems biology, nanobiotechnology, genome editing, phenomics and synthetic biology.

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

Submission The journal only accepts online submissions through the open journal system **(https://smujo.id/cbd/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. **Reprint** Authors or other parties may freely download and distribute. However, a printed request will be charged. It may be purchased when ordering by sending back the uncorrected proofs by email.

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

CELL BIOLOGY & DEVELOPMENT
Volume 7, Number 1, June 2023
Pages: 1-8

E-ISSN: 2580-4499
DOI: 10.13057/cellbioldev/v070101

Effect of cypermethrin insecticide on root chromosome morphometry of scallion (*Allium fistulosum*)

FESA AULIA NURUL HAQ, NITA ETIKAWATI, SOLICHATUN♥
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: 26 November 2022. Revision accepted: 2 February 2023.

Abstract. *Haq FAN, Etikawati N, Solichatun. 2023. Effect of cypermethrin insecticide on root chromosome morphometry of scallion (*Allium fistulosum*). Cell Biol Dev 7: 1-8.* Farmers in Tawangmangu, Karanganyar, Central Java, Indonesia, commonly use a pesticide known as cypermethrin insecticide whenever growing scallion (*Allium fistulosum* L.). If used at excessive levels, cypermethrin pesticide can be fatal to plants. Chromosome morphological changes are an indicator of cypermethrin insecticide's toxicity. Chromosomal morphometry, the study of chromosomal size and shape, can provide an overall picture of the quality of a plant's development. This research aimed to evaluate cypermethrin insecticide's impact on *A. fistulosum* root chromosomal morphometrics. Information gleaned from chromosomal morphometry includes chromosome shape, chromosome size, aberration type, chromosomal aberration index, relative asymmetry index, relative centromere index, and mitotic chromosome index. The concentration of cypermethrin was the sole independent variable in this study, which used a Completely Randomized Design (CRD) factorial arrangement. Long green onions were treated with varying concentrations of cypermethrin for up to four weeks: 0 mg/L, 0.05 mg/L, 0.10 mg/L, 0.15 mg/L, 0.20 mg/L, and 0.25 mg/L. The squash method was used to prepare the plant's apex roots. The root chromosomal visualization output was subjected to quantitative and qualitative descriptive analysis. The mitotic index decreased, and the chromosome aberration index increased after exposure to cypermethrin pesticides. The higher the concentration of the pesticide cypermethrin, the lower the mitotic index and the greater the chromosome aberration index. Long green onion root cells exposed to cypermethrin insecticide underwent chromosomal aberrations that could be identified qualitatively. Sticky chromosomes, chromosome bridge, chromosome agglutination, disrupted metaphase, disturbed anaphase, and hypoploid cell are examples of chromosomal aberrations.

Keywords: *Allium fistulosum,* chromosome, cypermethrin, mitosis, morphometry, scallion

INTRODUCTION

Karanganyar District, Central Java, Indonesia, is an area that acts as a center for producing horticultural crops and various other types of plants, such as food crops, ornamental plants, and medicinal plants. One flowering plant often found in agricultural areas in Tawangmangu, Karanganyar District, Central Java, is a scallion (*Allium fistulosum* L.). The *A. fistulosum,* or spring onion, is a plant usually used as a food ingredient. According to data from the Karanganyar Central Bureau of Statistics in 2018, *A. fistulosum* is one of the plants with the third largest commodity production after carrots and mushrooms in Karanganyar District.

The interviews show that most *A. fistulosum* farmers in the Tawangmangu, Karanganyar District area carry out many farming activities accompanied by applying insecticides. The application of insecticides or pest control substances is carried out to increase crop production both in quality and quantity. The type of insecticide that farmers often use is cypermethrin. Several farmers in the Tawangmangu area used different insecticide concentrations, ranging from 0.05 mg/L to 0.20 mg/L. Cypermethrin is a type of insecticide from the organophosphorus group which is known to be toxic to certain kinds of animals, such as armyworms, grasshoppers, fleas, and other pests that attack *A. fistulosum.*

According to Onuminya and Eze (2019), an increase in the concentration of cypermethrin insecticide in plants is known to increase the occurrence of chromosomal aberrations and reduce the mitotic index in the roots of garlic (*Allium sativum* L.). This condition allows for a similar effect to the cypermethrin insecticide on *A. fistulosum*. Therefore, an increase in chromosomal aberrations and a decrease in the mitotic index may indicate a cytotoxic effect of the cypermethrin insecticide given during plant growth.

The toxic properties of cypermethrin insecticide could have an unfavorable impact on plants if the application is not following the recommended dose, including on food crops such as *A. fistulosum.* The cypermethrin-type insecticide farmers use is usually applied without a standard usage concentration. Instead, it is determined based on the unit area or the number of individuals planted, so the farmers in Tawangmangu likely apply the cypermethrin insecticide excessively. Excessive exposure to insecticides can affect chromosomes in plant organs, resulting in changes in plants' morphological characters and physiological processes. Nofitahesti and Daryono (2016) stated that changes in chromosome characters in horticultural plants such as *A. fistulosum* can result in various ways, such as disrupting the cell division process.

Therefore, to determine the toxic effect of cypermethrin, it is necessary to study changes in the chromosomal plants' characteristics exposed to cypermethrin to concentration ranges commonly used by farmers. In analyzing chromosomal characteristics, plants from the genus Allium are generally used. Plants of the Allium genus have prominent characteristics and are easy to observe. Levan (1935) states that the chromosomes in the roots of plants of the Allium genus have large sizes and are easy to count and observe. *A. fistulosum* is a cultivated plant most likely to get excessive exposure to cypermethrin insecticides, so this plant can be used to observe chromosomes in this study.

Information related to the cytotoxicity of cypermethrin insecticide on the shape and size of the chromosomes of *A. fistulosum* is challenging. Therefore, research on the effect of cypermethrin on the root chromosomes of *A. fistulosum* using chromosome morphometry measurement is needed. The observed root chromosome morphometry consisted of chromosome shape, number, size, chromosomal aberrations, and mitotic index. Therefore, this study aimed; (i) determine the effect of cypermethrin insecticide on the root chromosome morphometry; (ii) to determine the effect of different concentrations of cypermethrin insecticide on the root chromosome morphometry of *A. fistulosum.*

MATERIALS AND METHODS

Materials

This research was carried out from August to December 2020 at the Biology Laboratory of FMIPA and the Green House of the Central Laboratory of Universitas Sebelas Maret, Surakarta. Central Java, Indonesia. The main materials used in this study were *A. fistulosum*, Rizotin insecticide (100 mg/L cypermethrin content), 0.2% colchicine solution, (colchicine + ethanol + distilled water), 2% acetoorcein dye (orcein stain powder + glacial acetic acid + distilled water), glacial acetic acid, 1 M hydrochloric acid, distilled water, and glycerin.

Research design

This study used a Completely Randomized Design (CRD) with 1 (one) factor at the planting stage of *A. fistulosum*, namely the concentration of cypermethrin insecticide. Cypermethrin insecticide was made into six different concentrations, namely 0 mg/L (control), 0.05 mg/L, 0.10 mg/L, 0.15 mg/L, 0.20 mg/L, and 0.25 mg/L. Each treatment was made of five replications.

Procedure

Preparation of test plants (planting)

Each *A. fistulosum* seedling was planted in a polybag with a soil-compost mixture of 1:1 and placed in the Green House of Universitas Sebelas Maret. The seeds used the following criteria: (i) Seedlings were obtained from seeds that had been sown for ±4 weeks. (ii) Seedlings have no insecticide history application during seeding. (iii) Seedlings are planted in a planting medium in the form of soil mixed with compost in a ratio of 1:1. (iv) The *A. fistulosum* plant media is watered regularly every two days until the plants are ± eight weeks old or ready to be harvested.

Insecticide treatment

Insecticide treatment used six different concentrations, namely 0 mg/L (the control), 0.05 mg/L, 0.10 mg/L, 0.15 mg/L, 0.20 mg/L, and 0.25 mg/L. Insecticide formulations were sprayed on *A. fistulosum* from the 5^{th} to the 8^{th} week of age. Cypermethrin insecticide was applied on all parts of the plant above the ground using a 1 mL spray bottle for each test plant. The intensity of one time per week and in the afternoon.

Chemical manufacture

Colchicine solution 0.2%. Colchicine 0.2 g dissolved in 5 mL ethanol, then stirred until dissolved. After dissolving, 95 mL of distilled water was added and stirred until thoroughly mixed. The solution is stored in glass bottles.

Glacial acetic acid 45%. The 45% glacial acetic acid was prepared by mixing 45 mL of pure glacial acetic acid with 55 mL of distilled water, then stirring until smooth. The solution was stored in a dark bottle and at room temperature.

Acetoorcein 2% dye. Aceto orcein 2% is prepared by heating 45 mL of glacial acetic acid to almost boiling (90-100°C). After that, it is mixed with 2 grams of orcein powder and boiled for 10 minutes while continuing to stir. The solution was left to cool to room temperature, and then 55 mL of distilled water was added and stirred again until evenly distributed. Finally, the acetoorcein dye solution was filtered and stored in a dark bottle at room temperature.

1M HCl. Hydrochloric acid (HCl) 1 M is prepared from diluted 2 M HCl. Dilution was performed by mixing 50 mL of 2 M HCl with 50 mL of distilled water (1:1 ratio). The solution was stored in a dark bottle and at room temperature.

Chromosome preparation

Root chromosome preparations were made using the semi-permanent squash method. In the pre-treatment process, the root tips were cut 0.5-1.0 cm, soaked with 0.2% colchicine in a flacon bottle, and incubated in the refrigerator at 4°C for 4 hours. The root samples were then washed with distilled water three times. Following the fixation process, the root samples were soaked in 45% glacial acetic acid and incubated in the refrigerator at 4°C for 15 minutes. The root samples were then washed with distilled water three times. After hydrolysis, the root samples were soaked in 1M HCl and incubated in an oven at 60°C for 5 minutes. The root samples were then washed with distilled water three times. The next stage was the coloring process; the root samples were soaked in 2% acetoorcein dye solution for 1 hour and stored at room temperature (±25°C). After that, the root samples were taken and placed in glass objects. The root sample was dripped with glycerin and covered with a glass cover. The glass preparation is placed on a flat plane, then pressed (squash) or tapped gently until the sample is evenly crushed. The glycerin oozing out of the edges of the coverslip was cleaned, then sealed with clear nail polish.

Chromosome observations

Observations of root chromosome morphometry were performed using a light microscope. The ocular lens magnification was 100x, and the objective lens magnification was 10x (total magnification 1000x). Moreover, to improve the resolving power, immersion oil is used on the surface of the preparation. Preparations that can be observed are visualized with a digital microscope and the NIS-Elements F3.0 program to be documented in photographs. Sahin and Koca's (2018) observations of the visualization results of root chromosomes were carried out in 500 cells for each treatment sample. Photos of visualization results are observed and determined if chromosomal aberrations occur. Chromosomal aberrations can be in the form of laggard chromosomes, c-mitotic, chromosome bridges, and others.

Chromosome measurement

The results of the chromosome visualization were processed again with ImageJ Fiji version 1.51h to calculate the number and size of each chromosome arm. A total of five cells that were still actively dividing at the prometaphase stage from each treatment sample were selected to measure chromosomal characters. First, the number of chromosomes was counted manually per unit cell, then the size of the chromosomes was measured in micrometers (µm). Chromosome sizes include long arms, short arms, and the entire chromosome body.

Calculation of chromosome morphometry data

The results of the chromosome size calculation were then processed again to determine the quantitative data of chromosome morphometry. The result is in the form of relative centromere index (Ci%), relative asymmetry index (AsI%), total aberration index (TA%), and mitotic index (IM%). The calculation formula used:

Relative centromere index (Ci%)

$$Ci\% = \frac{short\ sleeve\ size}{total\ sleeve\ size} \times 100$$

Relative asymmetry index (AsI%)

$$AsI\% = \frac{long\ sleeve\ size}{total\ sleeve\ size} \times 100$$

Total aberration index (TA%)

$$TA = \frac{\text{the number of cells experiencing aberration}}{\text{the total number of dividing cells}} \times 100$$

Mitotic index (IM%)

$$IM = \frac{\text{the number of cells in the mitotic phase}}{\text{the total number of cells observed}} \times 100$$

Data analysis

Descriptive qualitative and quantitative are used to analyze data in this research. The observed data of chromosomal aberrations are described qualitatively. The morphometric data was then processed by calculating the number of chromosomes for each cell, the size, and the shape of chromosomes. The chromosomal morphometry data were analyzed using the SPSS 16.0 program through the DMRT test of one-way Analysis of Variance (OneWay ANOVA) with a 95% confidence level to assess the effect of the treatment factors.

RESULTS AND DISCUSSION

Chromosome morphology

Qualitative and quantitative characterization of *A. fistulosum* root chromosomes was conducted to detect changes in chromosome characters after being treated with insecticides during its growth period. Qualitatively, the type of chromosomal aberration can be determined when the cell is actively dividing. Setyawan and Sutikno (2000) stated that in Allium plants is more apparent at the prometaphase stage. This is because the location of the chromosomes would be more spread out, and the shape of the centromere indentation is more apparent at the prometaphase stage. In observing the sample without treatment (the control), some cells are still active in the prometaphase division phase. These cells are considered representative cells to show normal chromosomal characteristics when actively dividing cells without the effect of the treatment given (Figure 1).

In the process of dividing at the prometaphase stage, visualizing cells is then made. A karyotype map and details of the morphological characters of the chromosomes are carried out. Arsal (2018) stated karyotype map shows how many chromosomes are present in a cell with some details of the structure of the chromosomes and the number of chromosomes. For example, *a. fistulosum* is known to have a diploid number of chromosomes (2n) with eight pairs of homologous chromosomes. Suminah et al. (2002) stated that most cultivated onion plants are diploid with a primary chromosome number of eight (x=8), so 2n=16. The preparation and results of the karyotype map from normal representative cells without treatment (the control) in this study are shown in Figures 2 and 3.

Moreover, to determine the presence of chromosomal abnormalities in a cell, it is necessary to have a quantitative analysis by calculating the relative centromere index (Ci%) and relative asymmetry index (AsI%). According to Qurniawan et al. (2012), the calculation of the relative centromere index (Ci%) and relative asymmetry index (AsI%) requires that the chromosome size is known by measuring the length of the chromosome arms. Therefore, chromosome measurements from each treatment sample yielded relative centromere index (Ci%) and relative asymmetry index (AsI%) values, presented in Table 1.

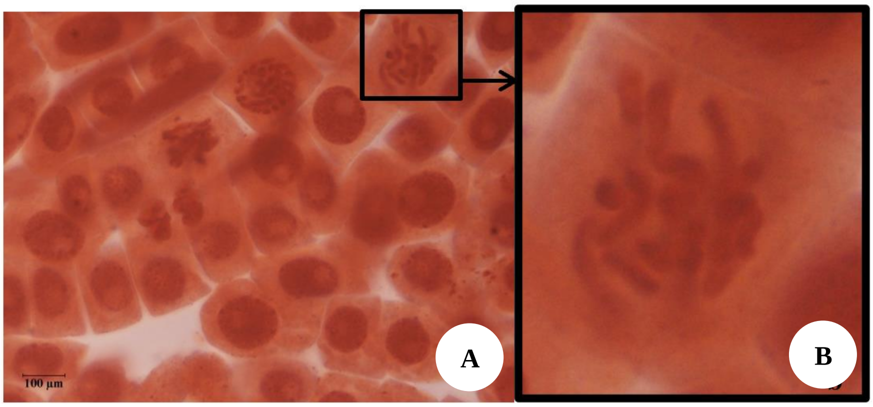

Figure 1. Results of observing the root cell chromosomes of *Allium fistulosum.* Note: A. magnification 1000x, B. Prometaphase stage of cell division

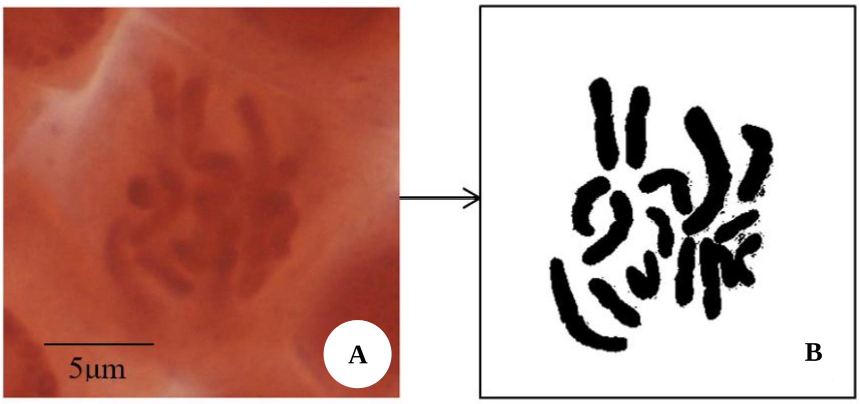

Figure 2. The results of observing the root cell chromosomes of *Allium fistulosum* at the prometaphase stage. Note: A. Prometaphase root cells, B. Chromosome schematic drawing

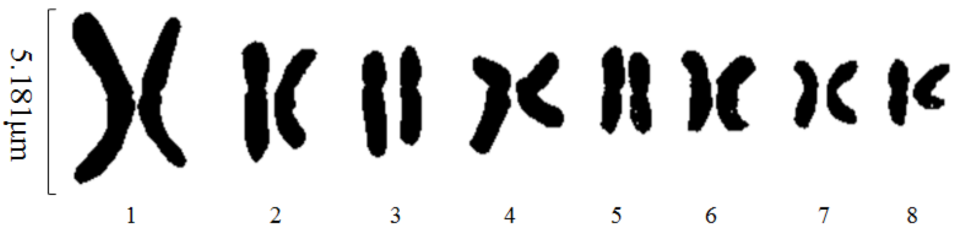

Figure 3. Karyotype map of *Allium fistulosum*

Table 1. The effect of different concentrations of cypermethrin insecticide on the quantitative character of the root chromosomes of *Allium fistulosum*

Cypermethrin concentration	$\bar{x}_p$	Ci%	AsI%
0 mg/L	3.59 µm	39.68	60.31
0.05 mg/L	5.84 µm	38.56	61.43
0.10 mg/L	6.01 µm	37.88	62.11
0.15 mg/L	5.10 µm	41.26	58.73
0.20 mg/L	4.43 µm	41.30	58.69
0.25 mg/L	5.48 µm	39.45	60.54

Note: The average value of total chromosome arm length (p), relative centromere index. (Ci%), and relative asymmetry index (AsI%)

Based on Table 1, the concentration of cypermethrin insecticide was assessed as not affecting the number and shape of the root chromosomes of *A. fistulosum.* The result shows that each difference in the concentration of cypermethrin gave normal levels. That same result as in the samples without cypermethrin insecticide treatment (the control). The six treatments with different concentrations had the same number of chromosomes, namely 2n = 16, and the chromosome pairs were dominated by metacentric chromosomes (Ci% = 37.50-50.00). The relative asymmetry index (AsI%) results indicate that the chromosomes are asymmetric in shape because there are also many sub-metacentric and telocentric chromosomes.

Setyawan and Sutikno (2000) suggested that a relative asymmetry index with a value of >50% indicated that symmetrical (metacentric) chromosomes were not dominant. In each sample, all chromosomes can be found in metacentric, sub-metacentric, and telocentric.

Mitotic index and chromosomal aberration index

The root mitotic index of *A. fistulosum* was influenced by the administration of cypermethrin insecticide and concentration changes, as determined by statistical analysis. The mitotic index value is the percentage of cells that undergo division in the observed samples. The mitotic index was often higher in the control group than in the cypermethrin-treated group. Likewise, cypermethrin differences are the higher the concentration, the lower the average mitotic index. The sample with the highest concentration of cypermethrin insecticide (0.25 mg/L) had the lowest mitotic index value (3.24%), whereas the sample without treatment (the control) had the highest mitotic index value (14.8%) (Figure 4).

The existence of cytotoxic characteristics generated by cypermethrin insecticide on *A. fistulosum* is evidenced by a decrease in the mitotic index. That is directly proportionate to an increase in the concentration of cypermethrin insecticide. It is consistent with the findings of Yakeen and Adeboye (2013), who noted that cypermethrin is a commonly used pyrethroid class insecticide for the upkeep of cultivated plants. Multiple studies have shown cypermethrin insecticides to cause significant chromosomal aberrations and mitotic division inhibition. Sahin and Koca (2018) added that as the mitotic index value drops, followed by mitotic activity drops along with it, which signals that DNA synthesis is being suppressed in the cell. There is speculation that the pesticide cypermethrin's cytotoxic characteristics could slow down DNA replication.

This study shows that cypermethrin pesticide is thought to cause chromosomal abnormalities and a drop in the mitotic index in the roots of *A. fistulosum*. The chromosomal aberration index measures the frequency of chromosomal abnormalities in actively dividing cells. Therefore, *a. fistulosum* roots were analyzed quantitatively to determine the chromosomal aberration index, and the findings are depicted in Figure 5.

The root aberration index of *A. fistulosum* was found to be affected by both the application of cypermethrin insecticide and concentration variations. Cypermethrin insecticide was found to have cytotoxic effects on actively proliferating *A. fistulosum* root cells, as evidenced by increased chromosomal aberration index value at higher dosages. The degree of chromosomal abnormalities was lower in the 0.25 mg/L cypermethrin insecticide treatment than in the 0.20 mg/L treatment. That treatment shows cells in the 0.25 mg/L treatment still in their cell cycle's interphase and prophase stages. Due to the slowed rate of cell division, abnormalities cannot be detected. According to studies by Chandraker et al. (2014), measuring the extent to which interphase cell division is dominant can indicate mitotic cycle suppression caused by hazardous chemicals.

Chromosomal aberrations

Based on qualitative observations of chromosomal aberrations, multiple types of aberrations were observed in *A. fistulosum* root cells from all samples, ranging from untreated samples to those treated with 0.25 mg/L of cypermethrin insecticide. Chromosomal aberrations are a qualitative description of cytological mutations in cells. Sahin and Koca's (2018) research indicates that a decrease in the mitotic index and the presence of chromosomal aberrations in the form of laggard chromosomes, micronuclei, and sticky chromosomes indicate the presence of cytotoxic properties of foreign substances. That also indicates the affected meristematic cells during division.

According to Rao et al. (2005), high quantities of cypermethrin cause chromosomal aberrations in metaphase and chromosome bridge creation in anaphase. In this research, sticky chromosomes (Figure 6); chromosome bridge (Figure 7); chromosome agglutination (Figure 8); disrupted metaphase (Figure 9), and disturbed anaphase were identified as chromosomal aberrations (Figure 10). The following explains the sticky chromosome, chromosome bridge, chromosome agglutination, disrupted metaphase, and disturbed anaphase.

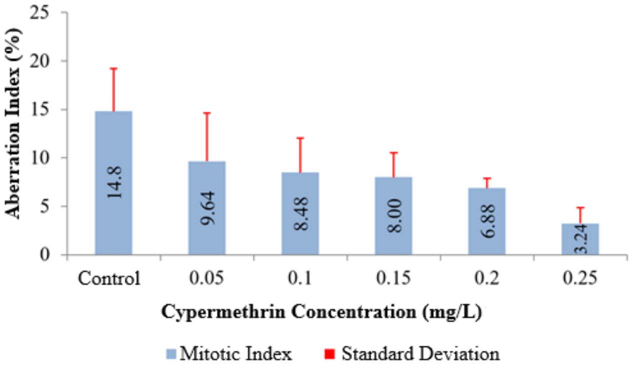

Figure 4. The relationship between the concentration of cypermethrin insecticide and the root mitotic index of *Allium fistulosum*

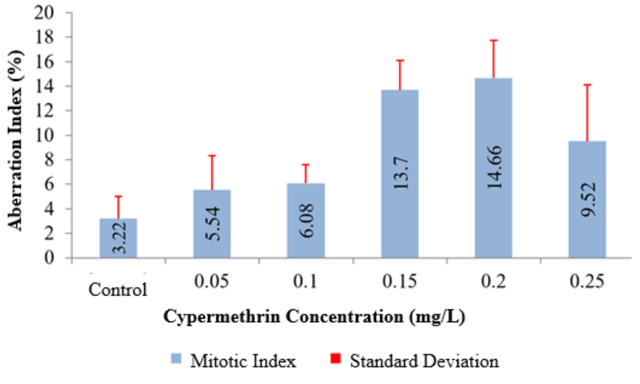

Figure 5. The relationship between the concentration of cypermethrin insecticide and the chromosomal aberration index of the roots of *Allium fistulosum*

A sticky chromosome is a type of chromosomal aberration characterized by the attachment of several chromosome numbers to one another, hence limiting mitotic division. According to Rosculete et al. (2019), the sticky chromosome is the most prevalent form of chromosomal aberration observed at the terminals of Allium roots. The aberration of sticky chromosomes represents a significant toxicity effect and can result in cell death. The comparatively high toxicity of pyrethroid group chemicals on Allium plant roots causes irreversible physical damage to the chromosomal proteins in the arrangement of the cell's chromosomes (Yakeen and Adeboye 2013). Figure 6 depicts the results of observations of sticky chromosomal aberration.

(ii) Chromosome bridge or chromosomal bridge is an anaphase-stage chromosomal aberration characterized by the presence of chromosome arms between the two planes of division. Figure 7 depicts the findings of observations of chromosomal bridge abnormalities. According to Imaniar and Pharmawati (2014), chromosome bridges can arise due to inversion (re-insertion) during cross-over. That inversion causes the inverted portion to generate a centromere, resembling a bridge to another chromosome. In addition, according to Yakeen and Adeboye (2013), a significant number of chromosome bridges indicate cytotoxic chemical damage.

(iii) Chromosome agglutination or chromosome stacking, specifically chromosomal aberrations in which the chromosomes in the nucleus overlap in one field while the other is vacant. Figure 8 depicts the findings of observations of chromosomal agglutination abnormalities. According to Imaniar and Pharmawati (2014), mutagens in the form of excessive pesticide chemicals cause the fragmentation of chromosomes, which subsequently accumulate in one place.

(iv) In the abnormal metaphase and anaphase phases, the disturbances form as a chromosomal abnormality. Disturbed metaphase is a condition in which the metaphase plate cannot form correctly due to faulty cell structure and location, whereas disturbed anaphase is a condition in which the spindle and microtubule orientation is disturbed. According to El-Araby et al. (2020), the primary cause of decreasing percentage of cell division is the disruption of metaphase and anaphase. As a result of the toxic properties of foreign nanoparticle material inserted between microtubules and affecting the mitotic division process in cells. Furthermore, disturbed metaphase and disturbed anaphase are cited as side effects. Figures 9 and 10 depict the result of observations on chromosomal abnormalities in disturbed metaphase and anaphase.

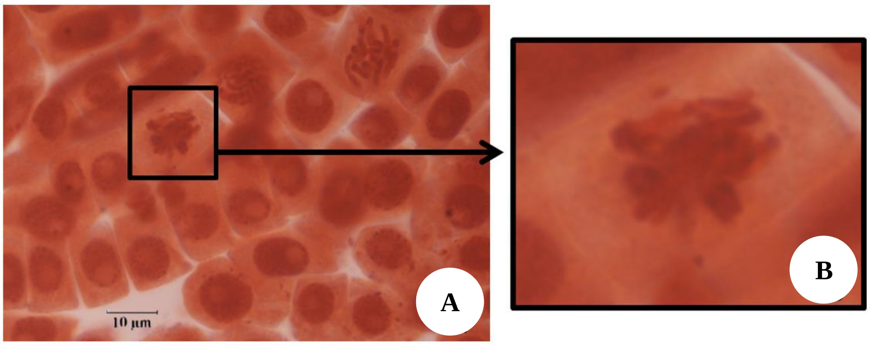

Figure 6. Results of observations of chromosomal aberrations in the root cells of *Allium fistulosum.* Note: A. magnification 1000x, B. sticky chromosome

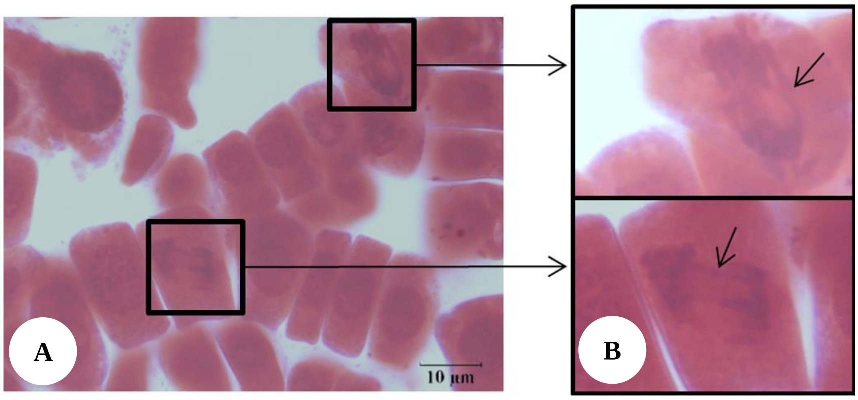

Figure 7. Results of observations of chromosomal aberrations in the root cells of *Allium fistulosum.* Note: A. magnification 1000x, B. chromosome bridge

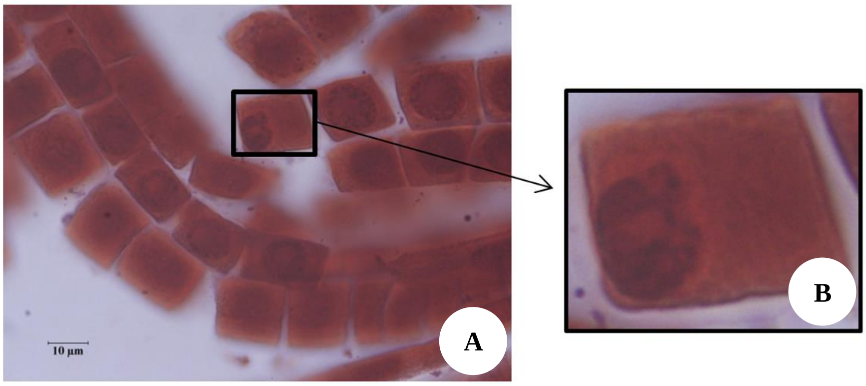

Figure 8. Results of observations of chromosomal aberrations in the root cells of *Allium fistulosum.* Note: A. magnification 1000x, B. chromosome agglutination

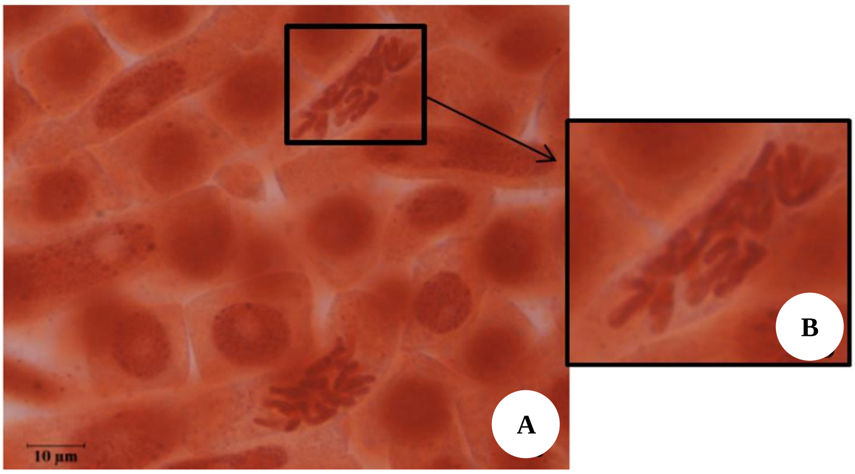

Figure 9. Results of observations of chromosomal aberrations in the root cells of *Allium fistulosum.* Note: A. magnification 1000x, B. disturbed metaphase

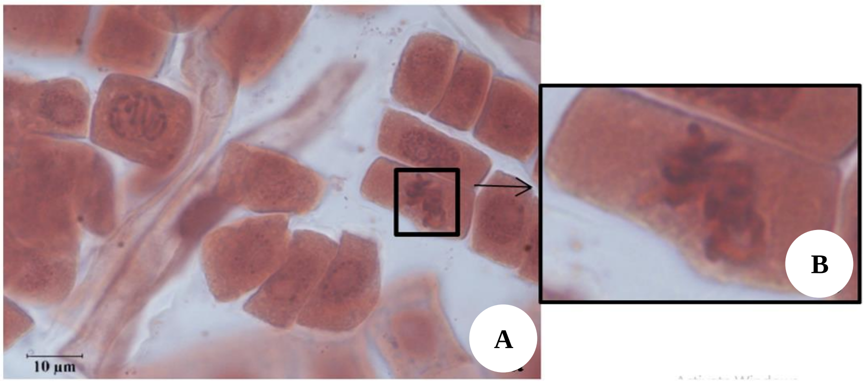

Figure 10. Results of observations of chromosomal aberrations in the root cells of *Allium fistulosum.* Note: A. magnification 1000x, B. disturbed anaphase

The presence of chromosomal abnormalities in the root cells of *A. fistulosum* suggests that cypermethrin insecticide has a direct influence on cell division and will have a negative effect on the growth of *A. fistulosum*. When used excessively, cypermethrin insecticides' toxicity can potentially affect cultivated plants' growth. The use of cypermethrin insecticides on *A. fistulosum* necessitates more specific instructions regarding the ideal concentration level for particular plant units to preserve proper plant growth.

Based on the provided results and discussion, the following conclusions are as follows; (i) The insecticide cypermethrin influences the root chromosomal morphometry of *A. fistulosum*. In response to the chromosomal index and chromosomal aberrations, the mitotic index value decreased, and the chromosomal aberration index value increased. Sticky chromosomes, chromosome bridge, chromosome agglutination, disrupted metaphase, and disturbed anaphase is examples of chromosomal abnormalities. (ii) Different cypermethrin insecticides dosage influence the chromosomal morphometry of *A. fistulosum* roots. The higher the concentration of the pesticide cypermethrin, the lower the mitotic index and the higher the chromosome aberration index.

REFERENCES

Arsal AF. 2018. Genetika 1 Arif Memahami Kehidupan. Badan Penerbit UNM, Malang. [Indonesian]

Chandraker SK, Singh P, Pandey B. 2014. Clastogenic effect of soft drink on root tip of *A. cepa*. Intl J Curr Microbiol Appl Sci 3 (5): 200-206.

El-Araby HG, El-Hefnawy SFM, Nassar MA, Elsheery NI. 2020. Comparative studies between growth regulators and nanoparticles on growth and mitotic index of pea plants under salinity. Afr J Biotechnol 19 (8): 564-575. DOI: 10.5897/AJB2020.17198.

Imaniar EF, Pharmawati M. 2014. Kerusakan kromosom bawang merah (*Allium cepa* L.) akibat perendaman dengan etidium bromida. Jurnal Simbiosis 2 (2): 173-183. [Indonesian]

Levan A. 1935. Cytological studies in *Allium*, VI the chromosome morphology of some diploid species of *Allium*. Hereditas 20 (3): 289-330. DOI: 10.1111/j.1601-5223.1935.tb03192.x.

Nofitahesti I, Daryono BS. 2016. Karakter fenotip kedelai (*Glycine max* (L.) Merr.) hasil poliploidisasi dengan kolkisin. Jurnal Sains dan Pendidikan Sains 5 (2): 90-98. [Indonesian]

Qurniawan TF, Arisuryanti T, Handayani NSN. 2012. Analisis kariotipe ular trawang (*Coelognathus radiatus*, (Boie 1827)). Jurnal Biologi Indonesia 8 (2): 247-254. [Indonesian]

Rao BV, Narasimham TL, Subbarao MV. 2005. Relative genotoxic effects of cypermethrin, alphamethrin and fenvalerate on the root meristems of *Allium cepa*. Cytologia 70 (3): 225-231. DOI: 10.1508/cytologia.70.225.

Rosculete CA, Bonciu E, Rosculete E, Olaru LA. 2019. Determination of the environmental pollution potential of some herbicides by the assessment of cytotoxic and genotoxic effects on *Allium cepa*. Intl J Environ Res Public Health 16 (75): 1-10. DOI: 10.3390/ijerph16010075.

Sahin Z, Koca S. 2018. Determination of the Genotoxicity of the soil in the Aydın Region Irrigated by Büyük Menderes River by the Allium Test System. Cumhuriyet Sci J 39 (1): 152-161. DOI: 10.17776/csj.405712.

Setyawan AD, Sutikno. 2000. Karyotipe kromosom pada *Allium sativum* L. (Bawang Putih) dan *Pisum sativum* L. (Kacang Kapri). Biosmart 2 (1): 20-27. [Indonesian]

Suminah, Sutarno, Setyawan AD. 2002. Induksi poliploidi bawang merah (*Allium ascalonicum* L.) dengan pemberian kolkisin. Biodiversitas 3 (1): 174-180. [Indonesian]

Onuminya TO, Eze TE. 2019. Cytogenotoxic effects of cypermethrin on root growth: *Allium sativum* as a model system. Asia-Pac J Mol Biol Biotechnol 27 (4): 54-61. DOI: 10.35118/apjmbb.2019.027.4.06.

Yakeen T, Adeboye MK. 2013. Cytogenotoxic effect of Cyermethrin, Deltamethrin, Lambdacyhalothrin, and Endosulfan Pesticides on *Allium cepa* root cells. African J Biotechnol 12 (41): 6000-6006. DOI: 10.5897/AJB2013.12802.

CELL BIOLOGY & DEVELOPMENT
Volume 7, Number 1, June 2022
Pages: 9-19

E-ISSN: 2580-4499
DOI: 10.13057/cellbioldev/v070102

Determination of active components from selected plants used in traditional medicine in Nigeria by GC-MS screening

MICHAEL SUNDAY ABU[1,♥], RUKAIYAT LAWAL MASHI[2], JAMILA YAHAYA LAWAL[2], ELIZABETH HABU EZEKIEL[1]

[1]Department of Biochemistry, Faculty of Pure and Applied Sciences, P.M.B 1020, Wukari, Federal University Wukari. Taraba State, Nigeria. Tel.: +23-470-68385043, ♥email: abulex77@yahoo.com
[2]Department of Biological Science, Federal College of Education. Dutsin-ma Road, Katsina Kastina-Nigeria

Manuscript received: 11 April 2023. Revision accepted: 13 June 2022.

Abstract. *Abu MS, Mashi RL, Lawal JY, Ezekiel EH. 2023. Determination of active components from selected plants used in traditional medicine in Nigeria by GC-MS screening. Cell Biol Dev 7: 9-19.* Herbal products have been utilized extensively in treating various ailments for centuries. Because of the enormous range of functionally relevant secondary metabolites of microbial and plant species, natural products and related structures are critical sources for discovering novel medications. The study investigated the phytoconstituents of extract of selected plants commonly used in traditional medicine in Nigeria, which include: bitter leaf (*Vernonia amygdalina* Delile), ginger (*Zingiber officinale* Roscoe), turmeric (*Curcuma longa* L.), bitter kola (*Garcinia kola* Heckel), Garlic (*Allium sativum* L.) and a mixture of all (turmeric, garlic, ginger, bitter leaf and bitter kola) using GC-MS (gas chromatography-mass spectrometry). GC-MS analysis was performed using Agilent 6890 GC with 59739N MSD and GC-MS equipped with Elite-I fused with silica capillary column (Cpsil 8cb: 30 mm x 25 mm x 0.25 mm). The carrier gas was Helium at a 1.5 mL/min flow rate. The sample was injected at a volume of 1 μL. The results of the analysis confirmed the presence of several compounds. The most prevailing compounds of medicinal value are Hexadecanoic acid, Cis-9,cis-12-Octadecadienoic acid, Squalene, Octanoic acid, Benzene, Pentanoic acid, beta.-Bisabolene, Nonanoic acid, aR-Turmerone, (3R,3aR,7R,8aS)-3,8,8-Trimethyl-6- methyleneoctahydro-1H-3a, Undecanoic acid, 2-Pentadecanone, Benzenedicarboxylic acid, Phytol, Linoleic acid, Dotriacontane, 1,3-Cyclohexadiene, 1,3-Cyclohexadiene, 2,6-Octadienal, 7-Propylidene-bicyclo4.1.0, Butan-2-one, 3-Cyclohexen-1-carboxaldehyde, (E)-1-(6,10-Dimethylundec-5-en-2-yl)-4-methylbenzene, 1-Decanol, Tetratetracontane, Caparratriene, Squalene, 9,19-Cyclolanost-24-en-3-ol. The evaluated plants showed the presence of various biologically active compounds as the scientific background of their use in traditional medicine.

Keywords: *Allium sativum, Curcuma longa, Garcinia kola,* GC-MS analysis, Polyherbal, *Vernonia amygdalina, Zingiber officinale*

INTRODUCTION

Plant products have been used in phytomedicines since ancient times (Cragg and David 2001; Belgica et al. 2021). Any plant part, such as the bark, leaves, flowers, roots, fruits, and seeds, may have active components. Herbal medicines are increasingly popular because they are safe, conveniently accessible, and have lower adverse effects (Ekor 2014). Many plants are less expensive and more accessible to most people, particularly in low-income countries, than modern medication, and there is a reduced rate of side effects following usage (Sofowora et al. 2013). These factors may account for their widespread interest and use. Some studies have documented the therapeutic benefits of some plants. Medicinal plants are the primary source of innovative medications and healthcare items (Ramalingum and Mahomoodally 2014). The extraction and characterization of various active phytocompounds from these green factories have developed certain medicines with high activity profiles (Sasidharan et al. 2011). Indeed, business and public demand for medicinal plants are so high that many therapeutic plants face a significant risk of extinction or loss of genetic diversity (Ekor 2014).

According to Mojab et al. (2003), understanding plant chemical ingredients is desirable since it will be useful in producing complicated chemical compounds. Previous studies have reported the phytochemical compounds of numerous plants. Several studies suggest that secondary plant metabolites may be nutritionally significant and play essential functions in human health (Lavecchia et al. 2013).

Sometimes, due to the synergistic effects, crude extract from medicinal plants is believed to have higher biological activity than isolated compounds (Jana and Shekhawat 2010). Plant phytochemical screening has revealed numerous chemicals, including alkaloids, flavonoids, tannins, steroids, glycosides, and saponins. Secondary plant metabolites are defense mechanisms against predation by many microorganisms, insects, and herbivores (Auwal et al. 2014).

Vernonia amygdalina Delile, belonging to the Asteraceae family, is a popular native vegetable in Nigeria, Uganda, and other African nations. It may be found in various biological zones across Africa and the Arabian Peninsula (Im et al. 2016). Phytochemicals extracted and isolated from *V. amygdalina* include saponins and alkaloids, terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, edotides, and sesquiterpenes (Farombi and Owoeye 2011).

Ginger (*Zingiber officinale* Roscoe) belongs to the family Zingiberaceae, originating from South East Asia. It is used in many countries as a spice and condiment to add flavor to food. Besides this, the rhizome of ginger has also been used in traditional herbal medicine (Mashhadi et al. 2013).

Curcuma longa L. is a member of the Zingiberaceae family and is widely cultivated in Asia's tropical regions. Its common name is turmeric root and yellow root. It has been used for centuries as a tonic and treats various ailments such as dyslipidemia, gastrointestinal issues, arthritis, and hepatic diseases (Mazzanti and Giacomo 2016).

Bitter kola (*Garcinia kola* Heckel) is a member of the Clusiaceae family, and Clusiaceae includes several significant fruit and medicinal tree species. Most Clusiaceae grow naturally or semi-domesticated but have been rediscovered as neglected or underutilized crops (Maňourová et al. 2019). Bitter kola is used in African ethnomedicine and traditional rituals.

Allium sativum L. is a member of the Liliaceae family that originated in Asia but is now grown in China, North Africa (Egypt), Europe, and Mexico. Various parts of the plant have long been employed in Iranian and other cultures' traditional folk remedies and as a spice and a food additive (Mikaili et al. 2013).

Plants have been employed as a source of medicine for many diseases since antiquity, owing to their variety and perhaps a rich complement of phytochemical and secondary metabolites (Kennedy and Wightman 2011). A wide range of herbs and herbal extracts contain various phytochemicals with biological activity that can be of valuable therapeutic index. Several phytochemicals have been discovered to have various medicinal properties, which may aid in preventing chronic diseases and metabolic diseases (Tungmunnithum et al. 2018).

In this research, some selected plants (*V. amygdalina, Z. officinale, C. longa, G. kola,* and *A. sativum*) that have been previously and currently being used for herbal decoction in Nigeria were evaluated for phytochemical constituents using Gas-Chromatography Mass Spectrometry (GC-MS).

MATERIALS AND METHODS

Collection and preparation of plant materials

Bitter leaf (*V. amygdalina*), ginger (*Z. officinale*), turmeric (*C. longa*), bitter kola (*G. kola*), garlic (*A. sativum*) were purchased from Wukari L.G.A, Taraba State, Nigeria. It was authenticated in the Department of Biochemistry, Federal University Wukari, Nigeria. The plant materials were thoroughly washed, chopped into pieces, and air-dried for five days before pulverization.

Extraction of plant materials

Exactly 200 g of the pulverized plant material was soaked in 500 mL ethanol for 24 hours at room temperature. Filtrate was filtered through Whatman No. 1 filter paper. After filtration, the extract was concentrated to dryness and kept in a refrigerator.

GC-MS (Gas Chromatography-Mass Spectrometry) analysis

GC-MS analysis was carried out on a Perkin Elmer Turbo Mass Spectrophotometer, which, including the column used, was a Perkin Elmer Elite-5 capillary column (30 m × 0.25 mm) with a film thickness of 0.25 mm composed of 95% Dimethylpolysiloxane. The carrier gas was Helium at a 1.5 mL/min flow rate. One µL sample was injected into the GC. The inlet temperature was maintained at 325°C. The oven temperature was programmed initially at 110°C for 4 min, then increased to 240°C. And after that, it was set to rise to 280°C at a rate of 20°C for 5 minutes. The total run time was 90 min.

The Mass Spectrometry transfer line was maintained at a temperature of 200°C. The source temperature was maintained at 180°C. Gas Chromatography-Mass Spectrometry was analyzed using electron impact ionization at 70 eV, and data were evaluated using Total Ion Count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of the known component spectrum in the GC-MS library. Measurement of peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

RESULTS AND DISCUSSION

The GC-MS chromatograms and the identified compounds in ethanol extract of some selected plants, i.e., bitter leaf (*V. amygdalina*), *g*inger (*Z. officinale*), turmeric (*C. longa*), bitter kola (*G. kola*), garlic (*A. sativum*) and their mixture are presented below:

In Table 1 and Figure 1, 1,3-Cyclohexadiene found in bitter cola has a peak area of 10.04%, while octanoic acid has the lowest peak area of 1.05%. The compound with the longest retention time (30.118 minutes) is Methoxyacetic acid; the shortest is Octanoic acid (5.93 minutes).

The compound with the highest peak area in the extract of bitter leaf is E-11-Hexadecenoic acid with a peak area of 14.00, while the lowest peak area of 0.38% corresponds to Undecanoic acid and Hexadecane, as shown in Table 2 and Figure 2. The retention times for Octanoic acid and Bis(2-ethylhexyl) phthalate compounds were 5.941 and 33.691 minutes, respectively.

Table 3 and Figure 3 show that the compound with the highest peak area in garlic extract is (E)-9-Octadecanoic acid, with a peak area of 16.74%, while Alpha-terpineol has the lowest peak area of 0.28%. (+)-Borneol and Dotriacontane had the shortest and the longest retention times at 5.35 and 29.42 minutes, respectively.

The compound with the highest peak area in the ginger extract was identified as Butan-2- one with a peak area of 12.29%, while the lowest peak area was (E)-1-(6,10-dimethyllundec-5-en-2-y-1)-4- methylbenzene (Table 4 and Figure 4). The longest retention time is 23.594 minutes ((E)-1-(6,10-Dimethylundec-5-en-2-y l)-4-methylbenzene), while the shortest is 5.363 minutes ((+)-Borneol).

Table 1. Identified compounds of ethanol extract of *Garcinia kola* by GC-MS analysis

RT (min)	Name	Mol. Formula	MW (g/mol)	Peak Area%
5.939	Octanoic acid	$\mathbf{C}_8H_{16}O2$	144.21	1.05
5.939	Benzhydrazide	$C_7H_8N_2O$	136.15	2.72
12.637	Benzene	C_6H_6	78.11	3.06
12.944	1,3-Cyclohexadiene	C_6H_8	80.13	10.04
13.263	N-Ethyl-p-toluidine	$C_9H_{13}N$	135.2062	2.99
13.616	Cyclohexene	C_6H_{12}	82.14	3.78
15.365	Decanoic acid	$C_{10}H_{20}O_2$	172.26	1.60
16.978	Ar-tumerone	$C_{15}H_{20}O$	216.387	15.53
17.345	Ar-tumerone	$C_{15}H_{20}O$	**216.387**	1.11
17.785	N-Ethyl-p-toluidine	$C_9H_{13}N$	135.2062	2.70
17.850	Sulfurous acid	H_2SO_3	82.07	2.82
20.594	1-Propanol	C_3H_80	60.09	1.12
20.809	Heptadecane	$C_{17}H_{36}$	240.5	0.83
21.360	Didodecyl phthalate	$C_{32}H_{54}O_4$	502.8	1.23
22.361	Heptadecane	$C_{17}H_{36}$	240.47	7.46
22.529	Heptadecane	$C_{17}H_{36}$	240.47	0.81
23.249	Dibutyl phthalate	$C_{16}H_{22}O$	278.34	5.75
23.884	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	2.15
26.494	N-[[2-p-Tolylsulfonyl]ethyl]phthalimide			6.02
26.993	Tritetracontane	$C_{43}H_{88}$	605.15	0.20
27.111	10-Octadecenoic acid	$C_{18}H_{34}O_2$	296.46	1.27
27.617	Octadecanoic acid	$C_{18}H_{32}O_2$	280.44	2.00
28.051	Methyl dithio phosphonic acid	$C_2H_7O_2PS_2$	158.17	2.73
28.792	1,8(2H,5H)-Isoquinoline dione	$C_9H_7NO_2$	161.16	1.12
30.021	3,5-Dimethylphenol	$C_8H_{10}O$	122.16	2.53
30.118	Methoxyacetic acid	$C_3H_6O_3$	90.08	1.50

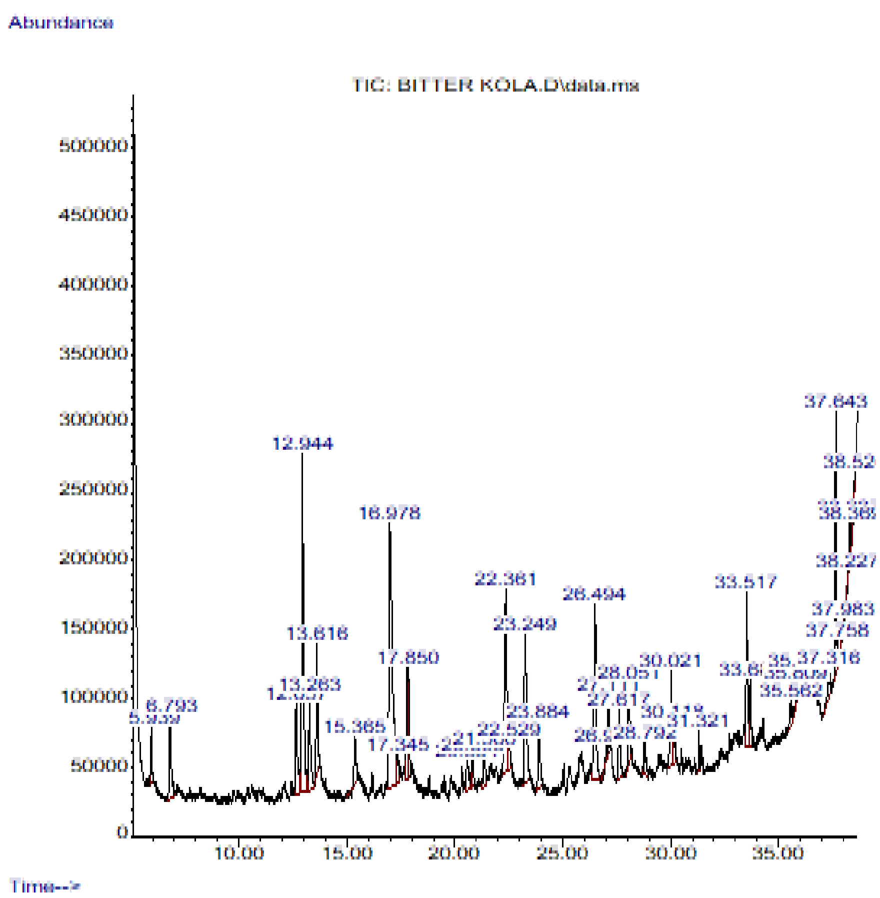

Figure 1. GC-MS Spectrum of ethanol extract of *Garcinia kola* Heckel

Table 2. Identified compounds of ethanol extract of *Vernonia amygdalina* by GC-MS

RT (min)	Name	Mol. Formula	MW (g/mol)	Peak Area%
5.941	Octanoic acid	$C_8H_{16}O2$	144.21	3.06
6.120	Decanal	$C_{10}H_{20}O$	156.26	1.75
12.644	3-Pyridinecarbonitrile	$C_6H_4N_2$	120.11	1.45
12.954	1,3-Cyclohexadiene	C_6H_8	80.13	9.56
13.283	beta.-Bisabolene	$C_{15}H_{24}$	204.35	4.23
13.634	Cyclohexene	C_6H_{10}	82.143	4.39
15.479	Undecanoic acid	$C_{11}H_{22}O_2$	186.29	4.98
19.886	Eicosanoic acid	$C_{20}H_{40}O_2$	312.53	0.40
19.923	Undecanoic acid	$C_{11}H_{22}O_2$	186.29	0.38
20.710	18-Nonadecen-1-ol	$C_{19}H_{38}O$	282.5	0.67
23.917	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	11.53
26.238	Phytol	$C_{20}H_{40}O$	296.53	1.72
26.405	4,8-Dioxatricyclo[5.1.0.0(3,5)]octane	$C_6H_8O_2$	112.13	0.95
27.051	9-Octadecynoic acid	$C_{18}H_{32}O_2$	280.4	2.27
27.150	E-11-Hexadecenoic acid	$C_{18}H_{34}O_2$	282.5	14.00
27.653	Undecanoic acid	$C_{11}H_{22}O_2$	186.29	4.48
31.429	1,2,5,6-Di-O-isopropylidene-3-O-me 1thanesulfonyl glucofuranose .alpha.-D-Glucopyranoside			0.67
32.740	Hexadecane	$C_{16}H_{34}$	226.41	0.38
33.524	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.55	1.86
33.691	Tartronic acid	$C_3H_4O_5$	120.06	0.90

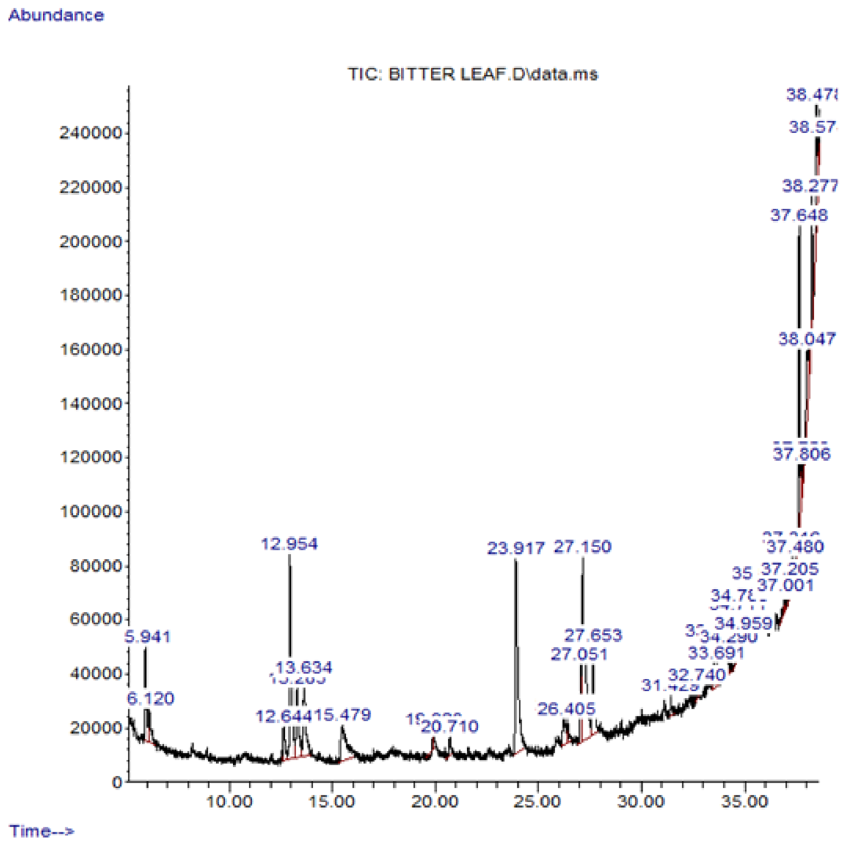

Figure 2. GC-MS Spectrum of ethanol extract of *Vernonia amygdalina* Delile

Table 3. Identified compounds identified of ethanol extract of *Allium sativum* L. by GC-MS

RT(min)	Name	Mol. Formula	M.W.(g/mol)	Peak Area%
5.359	(+)-Borneol Bicyclo[2.2.1]heptan-2-ol	$C_{10}H_{10}O$	154.25	1.14
5.871	.alpha.-Terpineol	$C_{10}H_{18}O$	154.25	0.28
5.940	Octanoic acid	$C_8H_{16}O_2$	144.21	1.31
12.647	Benzene	C_2H_6	78.11	1.66
12.945	Pentanoic acid	$C_5H_{10}O_2$	102.13	1.34
13.274	.beta.-Bisabolene	$C_{15}H_{24}$	204.35	0.97
13.625	(3R,3aR,7R,8aS)-3,8,8-Trimethyl-6-methylene octahydro-1H-3a	$C_{15}H_{24}$	204.35	1.09
15.381	Nonanoic acid	$C_9H_{18}O_2$	158.24	1.27
15.449	Pentanoic acid	$C_5H_{10}O_2$	102.13	0.29
16.988	aR-Turmerone	$C_{15}H_{20}O$	216.31	8.83
19.805	Undecanoic acid	$C_{11}H_{22}O_2$	186.29	1.37
20.839	2-Pentadecanone	$C_{15}H_{30}O$	226.39	0.86
22.536	Pentadecanoic acid	$C_{15}H_{30}O_2$	242.40	1.68
23.258	1,2-Benzenedicarboxylic acid	$C_8H_6O_2$	166.14	1.98
23.880	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	10.85
25.770	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280.45	0.80
25.876	7-Hexadecenoic acid	$C_{16}H_{28}O_2$	252.40	2.38
26.141	Phytol	$C_{20}H_{40}O$	296.53	1.05
27.001	Linoleic acid	$C_{18}H_{32}O_2$	280.45	3.67
27.114	(E)-9-Octadecenoic acid	$C_{18}H_{32}O_2$	280.4	16.74
27.625	Octadecanoic acid	$C_{18}H_{32}O_2$	280.44	3.63
29.428	Dotriacontane	$C_{32}H_{66}$	450.87	1.13

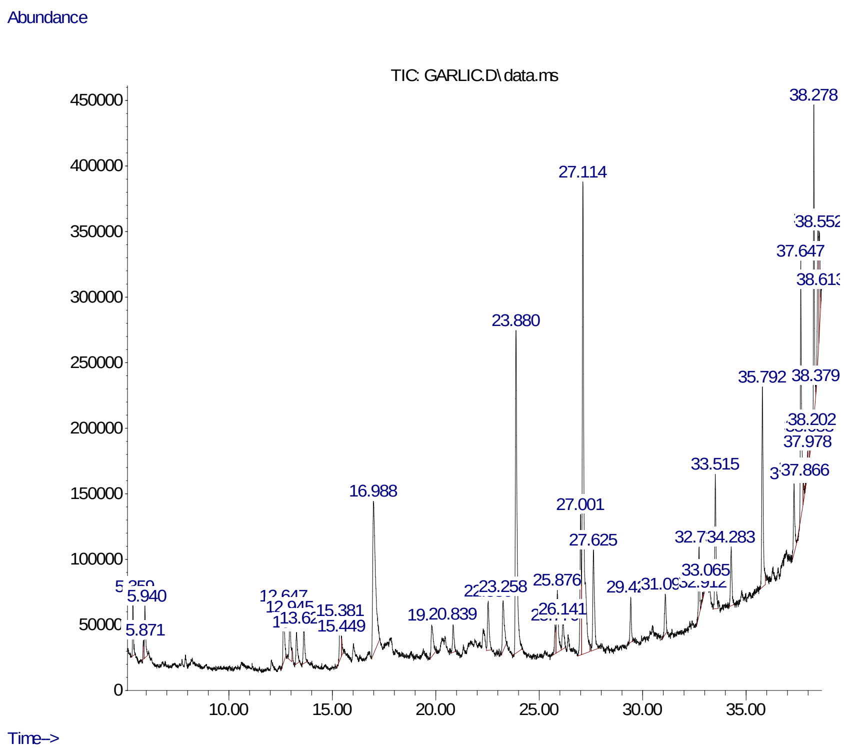

Figure 3. GC-MS Spectrum of ethanol extract of *Allium sativum* L.

Table 4. Identified compounds of ethanol extract of *Zingiber officinale* Roscoe by GC-MS

RT (min)	Name	Mol. Formula	MW (g/mol)	Peak Area%
5.363	(+)-Borneol	$C_{10}H_{18}O$	154.25	0.36
5.942	Octanoic acid	$C_8H_{16}O_2$	144.21	0.38
6.127	Decanal	$C_{10}H_{20}O$	156.26	3.84
12.554	Germacrene D			0.35
12.641	Benzene	C_2H_6	78.11	1.60
12.952	1,3-Cyclohexadiene	C_6H_8	80.12	9.76
13.272	1,3-Cyclohexadiene	C_6H_8	80.12	4.21
13.417	(E,Z)-.alpha.-Farnesene	$C_{15}H_{24}$	204.35	0.43
13.624	Cyclohexene	C_6H_{10}	82.143	4.14
14.265	Cyclohexanemethanol	$C_7H_{14}O$	114.19	0.28
14.351	Cyclohexane	C_6H_{10}	82.143	0.49
14.623	1,6,10-Dodecatrien-3-ol	$C_{15}H_{26}O_2$	222.36	0.41
15.238	cis-p-mentha-1(7)8 dien-2-ol	$C_{10}H_{16}O_2$	152.24	0.79
15.348	Dodecanoic acid	$C_{12}H_{24}O_2$	200.31	0.89
15.777	2,6-Octadienal	$C_8H_{12}O$	152.23	1.12
16.033	7-Propylidene-bicyclo[4.1.0]	$C_{10}H_{16}$	136.23	0.35
16.178	trans-Sesquisabinene hydrate	$C_{15}H_{26}O$	222.37	0.55
16.716	Butan-2-one	C_4H_8O	72.12	12.29
16.953	2-Butanone	C_4H_8O	72.12	6.21
17.486	2-Butanone	C_4H_8O	72.12	1.51
17.636	Butan-2-one	C_4H_8O	72.12	0.32
20.298	3-Cyclohexen-1-carboxaldehyde	$C_7H_{10}O$	110.15	0.81
23.594	(E)-1-(6,10-Dimethylundec-5-en-2-y l)-4-methylbenzene	$C_{20}H_{32}$	272.46	0.32

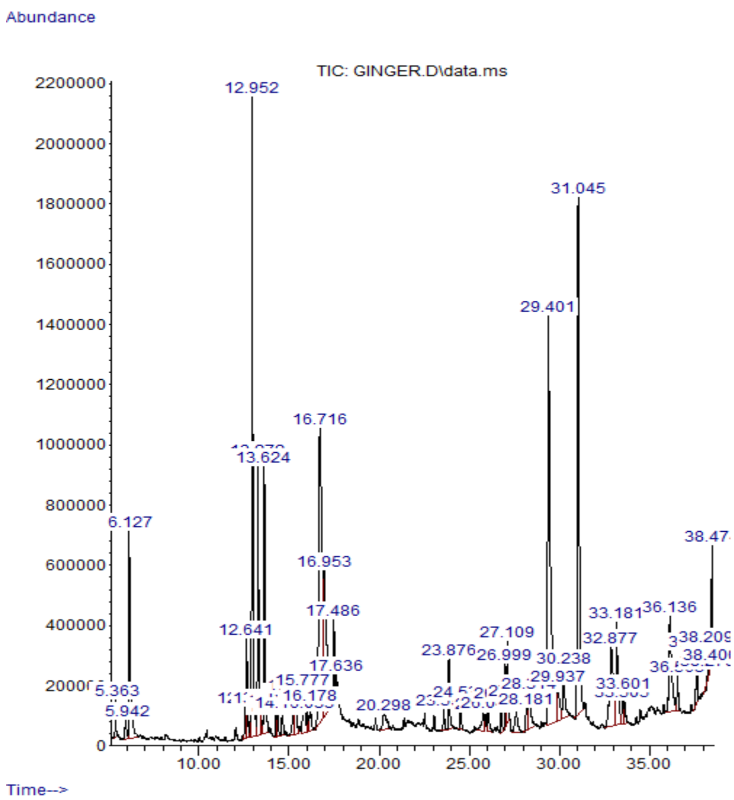

Figure 4. GC-MS Spectrum of ethanol extract of *Zingiber officinale* Roscoe

Table 5. Identified compounds of ethanol extract of *Curcuma longa* L. by GC-MS

RT (min)	Name	Mol. Formula	MW. (g/mol)	Peak Area%
23.945	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	1.13
27.177	E-11-Hexadecenoic acid	$C_{18}H_{34}O$	282.46	0.95
32.584	Cyclopropanemethanol	$C_7H_{14}O$	114.19	5.66
32.755	1,6,10,14,18,22-Tetracosahexaen-3-ol	$C_{30}H_{50}O$	426.71	4.41
32.755	Tetratetracontane	$C_{44}H_{90}O$	619.18	4.41
35.823	1-Decanol	$C_{10}H_{22}O$	158.28	0.39
36.370	9,19-Cyclolanost-24-en-3-ol	$C_{30}H_{50}O$	426.7	46.28
37.129	9,19-Cyclolanost-24-en-3-ol	$C_{30}H_{50}O$	426.7	3.48
37.655	Squalene	C30H50	410.73	1.01
38.339	Caparratriene	$C_{15}H_{26}$	207.37	21.64

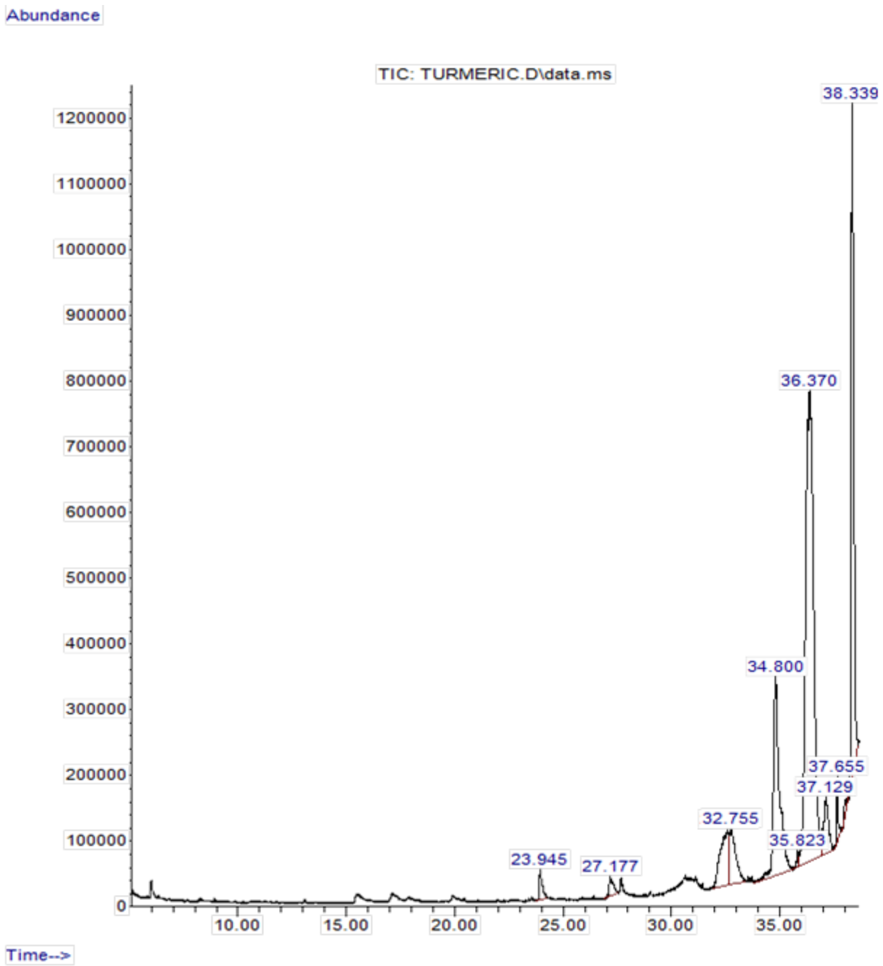

Figure 5. GC-MS Spectrum of ethanol extract of *Curcuma longa* L.

In turmeric, 9,19- Cycloanost-24-en-3-nol has the highest peak area of 46.28%, while 1-Decanol has the lowest peak area of 0.39% (Table 5 and Figure 5). Caparratriene exhibited the longest retention time of 38.339 minutes, while Hexadecanoic acid was eluded at 23.94 minutes.

Table 6 and Figure 6 show that Ar-tumerone has the highest peak area of 12.22% in the mixture of the plants, whereas 1,5- heptadiyne has the smallest peak area of 0.19%. The compound with the highest retention time is 13-Tetradecynoic acid methyl ester (22.542 minutes), while the lowest is 3-dimethyl- (5.39 minutes).

Table 6. Identified compounds ethanol extract of plant mixture by GC-MS

RT (min)	Name	Mol. Formula	MW. (g/mol)	Peak Area%
5.391	3-dimethyl-	C_6H_{12}	84.16	0.27
5.975	Octanoic acid	$C_8H_{16}O_2$	144.21	2.38
5.975	3-methyl-trans-2-Undecen-1-ol	$C_{11}H_{22}O$	70.29	2.38
6.252	methyl- 1-Decanol	$C_{11}H_{24}O$	172.31	0.36
12.649	8-Dimethyl-7-methylene octahydro-1H-3a	$C_{15}H_{22}O_2$	234.33	0.86
12.649	Carbonic acid	H_2CO_3	62.03	0.86
12.954	1,3-Cyclohexadiene	C_6H_8	80.12	5.44
13.286	4,11,11-trimethyl-8-methylene-	$C_{15}H_{24}$	204.35	1.43
13.359	trans-.alpha.-Bergamotene			0.14
13.634	Cyclohexene	C_6H_{10}	82.143	1.83
15.393	10-Bromodecanoic acid	$C_{15}H_{24}$	204.35	3.47
15.534	Octadecanoic acid	$C_{18}H_{32}O_2$	280.44	0.54
15.581	Decanoic acid	$C_{10}H_{20}O_2$	172.26	0.29
17.024	Ar-tumerone	$C_{15}H_{20}O$	216.31	12.22
17.332	Methanone	CO^-	28.010	0.31
17.812	2,4,6-trimethyl-phenol	$C_9H_{12}O$	138.19	3.74
19.401	1,5-Heptadiyne	C_7H_8	92.14	0.19
19.440	2,8-Decadiyne	$C_{10}H_{14}$	134.21	0.44
19.836	Octanoic acid	$C_8H_{16}O_2$	144.21	1.15
22.542	13-Tetradecynoic acid methyl ester	$C_{15}H_{26}O_2$	238.37	0.45

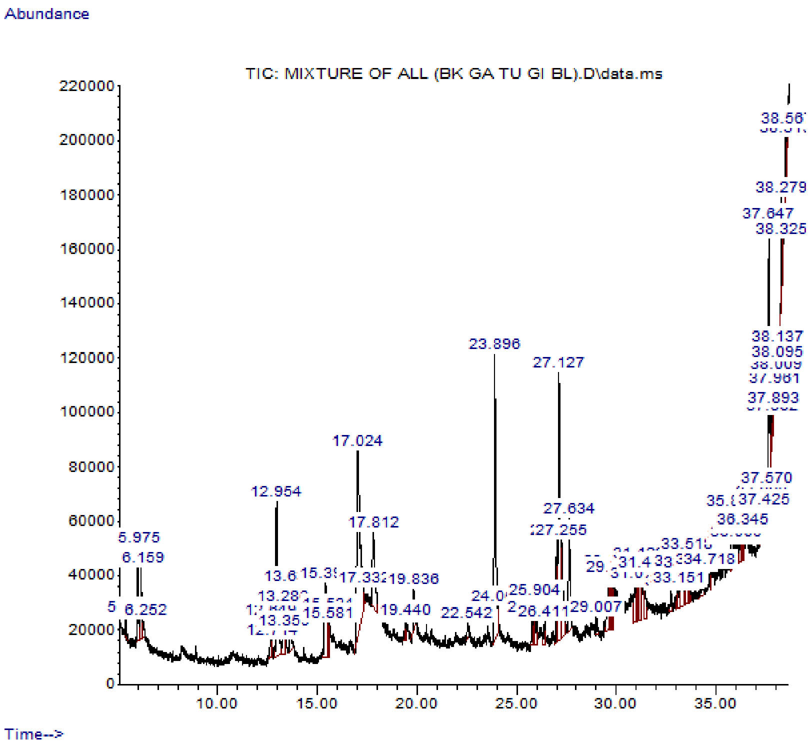

Figure 6. GC-MS Spectrum of ethanol extract of plant mixture

Discussion

Herbs have recently received global attention due to their products' advantages, especially for food and therapeutics (Ekor 2014). Plants bioactive components have various physiological effects, which are principally responsible for their medicinal properties (Altemimi et al. 2017). Essential oils, flavonoids, terpenoids, alkaloids, tannins, phenolic compounds, saponins, and cardiac glycosides are among the bioactive ingredients (Tungmunnithum et al. 2018).

The phytochemicals constituents of some selected plants such as; bitter leaf (*V. amygdalina*), ginger (*Z. officinale*), turmeric (*C. longa*), bitter kola (*G. kola*), garlic (*A. sativum*) and a mixture of all in the ratio of 1:1:1:1:1

(turmeric, garlic, ginger, bitter leaf, and bitter kola) are identified and presented Figures 1-6 and Tables 1-6). The presence of various bioactive compounds in the ethanol extract of the selected plants detected by GC-MS analysis justified the use of the whole plant for various ailments in traditional medicines.

The GC-MS spectrum of *G. kola* ethanol extract revealed 26 compounds (Table 1). The compounds present in the extracts of *Garcinia kola* include: octanoic acid, benzhydrazide, benzene, 1,3-cyclohexadiene, n-ethyl-p-toluidine, decanoic acid, cyclohexene, ar-turmerone, sulfurous acid and didodecyl phthalate, heptadecane, dibutyl phthalate, hexadecanoic acid, n-[[2-p-tolylsulfonyl] ethyl]phthalimide, tritetracontane, 10-octadecenoic acid, Octadecanoic acid, Methyl dithio phosphonic acid**,** 1,8(2H,5H)-Isoquinoline dione**,** 3,5-dimethylphenol, and methoxyacetic acid**.** Ar-turmerone is used in treating neurodegenerative diseases. Benzhydrazide is anticancer and antitumor, while Octanoic acid treats candidiasis and bacterial infections (Zappavigna et al. 2020). Methoxyacetic acid is a hazardous metabolite of ethylene glycol monomethyl ether frequently used as an industrial solvent (Bagchi and Waxman 2008). Janssen et al. (2004) found methoxyacetic acid to increase cellular sensitivity to estrogens, progestins, androgens, and other nuclear receptor hormone receptor ligands. Modulating nuclear receptor transcriptional activity by methoxyacetic acid usually proceeds through activating protein kinases and inhibiting histone deacetylases, which could lead to gonadal toxicities (Blainey et al. 2009).

On the other hand, Camphene (monoterpenes), 1,1,3,3-tetramethyl, Eucalyptol (1,8- cineole), Trans-caryophyllene, and Limonene were absent in the ethanol extract of *G. kola* in this study. Still, they were present in the Edo and Onoharigho (2022) study. On the contrary, results of the GC-MS analysis of the methanolic extract of *G. kola* (Rufa'I et al. 2023) showed the presence of cyclohexane, benzene, and hexanedioic (adipic) acid as present in the ethanol extract of *G. kol*a in this study. However, tridecanoic acid, tetrasiloxane, thymol, pentanone, and silane were absent. The phytochemical variation in the *G. kola* extract could be attributed to the ability of the different solvents to extract bioactive components, which depend on their polarity indexes.

GC-MS analysis of *V. amygdalina* ethanol extract showed 20 identified biologically active compounds (Table 2). The compounds include Octanoic acid, Decanal, 3-Pyridinecarbonitrile, 1,3-Cyclohexadiene, beta.-Bisabolene, Undecanoic acid, Undecanoic acid, Hexadecanoic acid, Phytol, 18-Nonadecen-1-ol. According to Addor (2017), undecanoic acid has antifungal properties, acts as a skin-protective antioxidant, and increases the amount of antioxidants in the skin. E-11-Hexadecenoic acid was the most abundant compound in the ethanolic extract of *V. amygdalina* leaf. This observation was contrary to the report of Alara et al. (2019) on ethanolic extract of *V. amygdalina* leaf, where Phytol was the most prevailing compound while E-11-Hexadecenoic acid was absent.

Table 3 shows the presence of 22 identified compounds as depicted by the GC-MS analysis of the ethanol extract of *A. sativum*. The identified compounds are; (+)-BorneolBicyclo[2.2.1]heptan-2-ol, alpha.-Terpineol, Octanoic acid, Benzene, Pentanoic acid, beta.-Bisabolene, 1,2-Benzenedicarboxylic acid, Hexadecanoic acid, 9,12-Octadecadienoic acid, 7-Hexadecenoic acid, E)-9-Octadecenoic acid, and Octadecanoic acid. In the present study, alpha-terpineol (0.28%) and pentanoic acid (0.29%) were present in modest concentrations in the *A. sativum* ethanol extract. In comparison, (E)-9-octadecenoic acid (16.74%) and hexadecanoic acid (10.85%) were the two predominant components. However, *A. sativum* ethanol extract reported by Park et al. (2017) presented higher concentrations of 2-propenoic acid, trisulfide, allyl trisulfide, 1,3-dihydroxyacetone dimer, acetaldehyde, and dihydroxyacetone. In another report, the extract's main constituents were trisulfide, di-2-propenyl (34.8%), and diallyl disulfide (14.83%) (Gong et al. 2021). The most prevalent non-sulfur components were acetic acid, 2-furan carboxaldehyde, and hexadecanoic acid (Badeli et al. 2022).

The GC-MS analysis (Table 4) of ethanol extract of *Z. officinale* indicates the presence of compounds with diverse biological activity, i.e.; (+)-Borneol, Octanoic acid, Decanal, Germacrene D, 1,3-Cyclohexadiene, (E)-1-(6,10-Dimethylundec-5-en-2-y-l)-4-methylbenzene, (E,Z)-.alpha.-Farnesene, Cyclohexanemethanol, 1,6,10-Dodecatrien-3-ol, and trans-Sesquisabinene hydrate. There is a variation of the compounds in ethanol extract. A previous study by Borekar et al. (2018) showed the presence of 12 major compounds that were identified as Isopropenyl dimethyl, 3-allyl-6-methoxyphenol, thujaketone, 2-butanoic acid, 2-methoxy methyl, hexahydro farnesol, gingerol, tetradecanoic acid, 1-phenyl-3-6-diazohomoadamantan-9-hydrozone, 7-methyl-2-tetradecen-1-ol-acetate, eicosane, androstane and squalene.

The GC-MS analysis of the ethanol extract of *C. longa* determined 10 identified compounds, as represented in Table 5. These compounds are Hexadecanoic acid, E-11-Hexadecenoic acid, Cyclopropanemethanol, 1,6,10,14,18,22-Tetracosahexaen-3-ol, Tetratetracontane, 9,19-Cyclolanost-24-en-3-ol, squalene. Squalene (triterpene) is a phenolic compound found in latex and resins of several plants, with the physiological function that is thought to be a defense mechanism against pathogens that cause human and animal diseases (Scortichini and Rossi 1991; Ezhilan and Neelamegam 2012). In the present study, the GC–MS analyses of the ethanolic *C. longa* extract revealed 46.28% of 9,19-Cyclolanost-24-en-3-ol as the most abundant compound and turmerone (7.14%); however, Abdel-Shafy et al. (2019) reported that the most abundant is turmerone. The presence of squalene reported in the present study was absent in studies by Singh et al. (2011) and Abdel-Shafy et al. (2019).

Lastly, GC-MS analysis of the mixture of the 5 plants showed the presence of some compounds, as shown in Table 6. Some of the compounds are; Methanone, 2,4,6-trimethyl-phenol, 1,5-Heptadiyne, 2,8-Decadiyne, Octanoic acid, 13-Tetradecynoic acid methyl ester, 3-dimethyl-, 3-

methyl-trans-2-Undecen-1-ol, 8-Dimethyl-7 methyleneoctahydro-1H-3. The presence of the compounds in the five plants mixture was known to be used as a flavoring agent, fungicide, pesticide, perfumery, anti-inflammatory, hypocholesterolemic, and cancer-preventive effects (Padma et al. 2019). However, the mixture of the five plants in the ratio of 1:1:1:1:1 does not necessarily increase the horizon of the bioactive components of it compared to the phytoconstituents of the individual plants.

The five plants and their mixture showed various secondary metabolites with relevant biological effects such as antioxidant, anticancer, antifungal, and anti-neurodegenerative activities. However, all the plants and the mixture except *C. longa* contain Octanoic acid, which has been known to have antioxidant activity. *A. sativum* and *Z. officinale* contained (+)-borneol, facilitating digestion and easing pain. Similarly, E-11-Hexadecenoic acid, an antioxidant, was found in *V. amygdalina* and *C. longa*.

Among the compounds present in the *A. sativum*, alpha-terpineol, octanoic acid, (+)-borneol, bicyclo[2.2.1]heptan-2-ol, pentanoic acid, aR-Turmerone, and undecanoic acid exhibit biological activities. Similarly, *Z. officinale* contained octanoic acid, (+)-borneol, germacrene D, and dodecanoic acid, which have biological activity. *Garcinia kola* contained bioactive compounds such as Octanoic acid, benzhydrazide, and Ar-tumerone. *V. amygdalina* contained octanoic acid, undecanoic acid, and E-11-hexadecenoic acid as secondary bioactive metabolites. Meanwhile, E-11-hexadecenoic acid and squalene were found in *C. longa*, Octanoic acid, and 2,8-Decadiyne in the mixture of the five plants as bioactive metabolites. The results revealed that more bioactive compounds were present in *A. sativum* compared to *Z. officinale, G. kola,* and *V. amygdalina*, which had fewer bioactive phytochemicals.

The findings showed the existence of components known to have pharmacological and physiological properties. This research demonstrated that these plants are excellent sources of bioactive chemicals with significant medical value. In conclusion, the phytochemical constituents of ethanol extract of some selected plants, i.e., bitter leaf (*V. amygdalina*), ginger (*Z. officinale*), turmeric (*C. longa*), bitter kola (*G. kola*), garlic (*A. sativum*) revealed the presence of various compounds that may have various biological activities.

ACKNOWLEDGEMENTS

Michael Sunday Abu designed and supervised the work, assisted in the lab work, prepared the manuscript, and provided technical support; Rukaiyat Lawal Mashicarried out data analysis and interpretation; Jamila Yahaya Lawal carried out manuscript proofreading and editing; and Oyebisi Sunday Samuel gave funding and carried out the lab work.

REFERENCES

Abdel-Shafy S, Alanazi AD, Gabr HSM, Allam AM, Abou-Zeina HAA, Masoud RA, Soliman DE, Alshahrani MY. 2019. Efficacy and safety of ethanolic *Curcuma longa* extract as a treatment for sand tampan ticks in a rabbit model. Vet World 13 (4): 812-820. DOI: 10.14202/vetworld.2020.812-820.

Addor FAS. 2017. Antioxidants in dermatology. An Bras Dermatol 92 (3): 356-362. DOI: 10.1590/abd1806-4841.20175697.

Alara OR, Abdurahman NH, Ukaegbu CI, Kabbashi NA. 2019. Extraction and characterization of bioactive compounds in *Vernonia amygdalina* leaf ethanolic extract comparing Soxhlet and microwave-assisted extraction techniques. J Taibah Univ Sci 13 (1): 414-422. DOI: 10.1080/16583655.2019.1582460.

Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. 2017. Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants 6 (4): 42. DOI: 10.3390/plants6040042.

Auwal MS, Saka S, Mairiga IA, Sanda KA, Shuaibu A, Ibrahim A. 2014. Preliminary phytochemical and elemental analysis of aqueous and fractionated pod extracts of *Acacia nilotica* (Thorn mimosa). Vet Res Forum 5 (2): 95-100.

Badeli Z, Haghkhah M, Ghaemi E A. 2022. Phytochemical analysis of garlic hydro-alcoholic extract and evaluation of its anti-bacterial effect on enterohemorrhagic *Escherichia coli* in vitro and ex vivo. Med Lab J 16 (1): 25-31.

Bagchi G, Waxman DJ. 2007. Toxicity of ethylene glycol monomethyl ether: impact on testicular gene expression. Intl J Androl 31 (2): 269-274. DOI: 10.1111/j.1365-2605.2007.00846.x.

Belgica THR, Suba MD, Alejandro CJD. 2021. Quantitative ethnobotanical study of medicinal flora used by local inhabitants in selected Barangay of Malinao, Albay, Philippines. Biodiversitas 22: 2711-2721. DOI: 10.13057/biodiv/d220720.

Blainey PC, Luo G, Kou SC, Mangel WF, Verdine GL, Bagchi B, Xie XS. 2009. Nonspecifically bound proteins spin while diffusing along DNA. Nat Struct Mol Biol 16 (12): 1224-1229. DOI: 10.1038/nsmb.1716.

Borekar AP, Somkuwar, RP Limsay, NV Kurkure, Bonde SW. 2018. Evaluation of active components from ethanolic extract of *Zingiber officinale* Rhizomes VI. J Pharmacog Phytochem 7 (4): 2779-2783.

Cragg GM, David JN. 2001. Natural product drug discovery in the next millennium. Pharm Biol 39: 8-17. DOI: 10.1076/phbi.39.s1.8.0009.

Edo GI, Onoharigho FO. 2022. Analysis of phytochemical constituents and antioxidant potential of bitter kola leaf extract towards bioactive food, nutrition and health resources. Organic Med Chem Intl J 11 (5): 555823. DOI: 10.19080/OMCIJ.2022.11.555823.

Ekor M. 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol 4: 1-10. DOI: 10.3389/fphar.2013.00177.

Ezhilan BP, Neelamegam R. 2012. GC-MS analysis of phytocomponents in the ethanol extract of *Polygonum chinense* L. Pharmacogn Res 4 (1): 11-4. DOI: 10.4103/0974-8490.91028.

Farombi EO, Owoeye O. 2011. Antioxidative and chemopreventive properties of *Vernonia amygdalina* and *Garcinia biflavonoid*. Intl J Environ Res Public Health 8 (6): 2533-55. DOI: 10.3390/ijerph8062533.

Gong X, Su X, Liu H. 2021. Diallyl Trisulfide, the antifungal component of garlic essential oil and the bioactivity of its nanoemulsions formed by spontaneous emulsification. Molecules 26 (23): 7186. DOI: 10.3390/molecules26237186.

Im E, Ae A, Bn U, Po U. 2016. Immuno-modulatory properties of prebiotics extracted from *Vernonia amygdalina*. Afr J Tradit Complement Altern Med 13 (6): 11-17. DOI: 10.21010/ajtcam.v13i6.3.

Jana S, Shekhawat GS. 2010. Phytochemical analysis and antibacterial screening of in vivo and in vitro extracts of indian medicinal herb: *Anethum graveolens*. Res J Med Plant 4: 206-212. DOI: 10.3923/rjmp.2010.206.212.

Janssen I, Krabbendam L, Bak M, Hanssen M, Vollebergh W, de Graaf R, van Os J. 2004. Childhood abuse as a risk factor for psychotic experiences. Acta Psychiatr Scand 109 (1): 38-45. DOI: 10.1046/j.0001-690x.2003.00217.x.

Kennedy DO, Wightman EL. 2011. Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. Adv Nutr 2 (1): 32-50. DOI: 10.3945/an.110.000117.

Lavecchia T, Rea G, Antonacci A, Giardi MT. 2013. Healthy and adverse effects of plant-derived functional metabolites: The need of revealing their content and bioactivity in a complex food matrix. Crit Rev Food Sci Nutr 53 (2): 198-213. DOI: 10.1080/10408398.2010.520829.

Maňourová A, Leuner O, Tchoundjeu Z, Van Damme P, Verner V, Přibyl O, Lojka B. 2019. Medicinal potential, utilization and domestication status of bitter kola (*Garcinia kola* Heckel) in West and Central Africa. Forests 10 (2): 124. DOI: 10.3390/f10020124.

Mashhadi NS, Ghiasvand R, Askari G, Hariri M, Darvishi L, Mofid MR. 2013. Anti-oxidative and anti-inflammatory effects of ginger in health and physical activity: review of current evidence. Intl J Prev Med 4 (Suppl 1): S36-42.

Mazzanti G, Di Giacomo S. 2016. Curcumin and resveratrol in the management of cognitive disorders: What is the clinical evidence? Molecules 21 (9): 1243. DOI: 10.3390/molecules21091243.

Mikaili P, Maadirad S, Moloudizargari M, Aghajanshakeri S, Sarahroodi S. 2013. Therapeutic uses and pharmacological properties of garlic, shallot, and their biologically active compounds. Iran J Basic Med Sci 16 (10): 1031-1048.

Mojab F, Kamalinejad M, Ghaderi N, Vahidipour HR. 2003. Iran J Pharm Res 3: 77-82. DOI: 10.22037/ijpr.2010.16.

Padma M, Ganesan S, Jayaseelan T, Azhagumadhavan S, Sasikala P, Senthilkumar S, Mani P. 2019. Phytochemical screening and GC–MS analysis of bioactive compounds present in ethanolic leaves extract of *Silybum marianum* (L). J Drug Deliv Ther 9 (1): 85-89. DOI: 10.22270/jddt.v9i1.2174.

Park N, Lee S, Boby N, Park S. 2017. GC/MS analysis, antimicrobial and antioxidant effect of ethanol garlic extract. Intl J Phytomed 9: 324-331. DOI: 10.5138/09750185.2087.

Ramalingum N, Mahomoodally MF. 2014. The therapeutic potential of medicinal foods. Adv Pharmacol Sci 2014: 354264. DOI: 10.1155/2014/354264.

Rufa'i FA, Baecker D, Mukhtar MD. 2023. Phytochemical screening, GC-MS analysis, and evaluating in vivo antitrypanosomal effects of a methanolic extract of *Garcinia kola* nuts on rats. Antibiotics 12 (4): 713. DOI: 10.3390/antibiotics12040713.

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L. 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med 8 (1): 1-10. DOI: 10.4314/ajtcam.v8i1.60483.

Scortichini M, Rossi MP. 1991. Preliminary in vitro evaluation of antimicrobial activity of terpenes and terpenoids towards *Erwinia amylovora* (Burrill) Winslow et al. J Appl Bacterial 71: 109-112. DOI: 10.1111/j.1365-2672.1991.tb02963.x.

Singh S, Sankar B, Rajesh S, Sahoo K, Subudhi E, Nayak S. 2011. Chemical composition of turmeric oil (*Curcuma longa L.* cv. Roma) and its antimicrobial activity against eye infecting pathogens. J Essent Oil Res 23 (6): 11-18. DOI: 10.1080/10412905.2011.9712275.

Sofowora A, Ogunbodede E, Onayade A. 2013. The role and place of medicinal plants in the strategies for disease prevention. Afr J Tradit Complement Altern Med 10 (5): 210-229. DOI: 10.4314/ajtcam.v10i5.2.

Tungmunnithum D, Thongboonyou A, Pholboon A, Yangsabai A. 2018. Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: An overview. Medicines 5 (3): 93. DOI: 10.3390/medicines5030093.

Zappavigna S, Cossu AM, Grimaldi A, Bocchetti M, Ferraro GA, Nicoletti GF, Filosa R, Caraglia M. 2020. Anti-inflammatory drugs as anticancer agents. Intl J Mol Sci 21 (7): 2605. DOI: 10.3390/ijms21072605.

CELL BIOLOGY & DEVELOPMENT
Volume 7, Number 1, June 2023
Pages: 20-27
E-ISSN: 2580-4499
DOI: 10.13057/cellbioldev/v070103

Comparing in vitro plant regeneration ability of *Oryza sativa* L. cv. Fujisaka 5 and *Brachiaria decumbens* from embryogenic callus

ZAINAH DAUD[1], AHMAD SOFIMAN OSMAN[1], NADALI B. JELODAR[2], LAI-KENG CHAN[1,3,♥]
[1]School of Biological Sciences, Universiti Sains Malaysia. 11800 Penang, Malaysia
[2]College of Agriculture, Sari Agricultural Sciences & Natural Resources University. Mazandaran, Iran
[3]D'Arboretum. 505-1, Batu 7, Jalan Masjid, Bukit Rambai 75250 Melaka, Malaysia.♥email: merrilynchanboey@gmail.com

Manuscript received: 29 May 2023. Revision accepted: 21 June 2023.

Abstract. *Daud Z, Osman AS, Jelodar NB, Chan L-K. 2022. Comparing in vitro plant regeneration ability of* Oryza sativa *L. cv. Fujisaka 5 and* Brachiaria decumbens *from embryogenic callus. Cell Biol Dev 7: 20-27.* The aim of this study was to compare the plant regeneration ability of *Oryza sativa* L. cv Fujisaka 5, a cold resistance japonica rice and *Brachiaria decumbens* Stapf, a tropical savanna grass, using embryogenic calli. The plant regeneration ability of both species has not been reported elsewhere. Friable calli were induced from the *O. sativa* Fujisaka 5 seeds on MS medium supplemented with 2.0 mgL^{-1} 2,4-D. While embryogenic calli were produced from *B. decumbens* seeds using a similar culture medium and remained embryogenic even after frequent subculturing. The friable calli of *O. sativa* Fujisaka 5 became embryogenic (88.2-97.7%) when they were subcultured onto MS medium containing 2,4-D and kinetin or BAP and NAA (0.5-1.0 mgL^{-1}) for four weeks. When the induced embryogenic calli (0.5 g) of both species were subcultured onto MS without plant growth regulators, plantlets were generated after one to two weeks with the formation of 2-3 plantlets for *O. sativa* Fujisaka 5 and 4-5 plantlets for *B. decumbens* per 0.5 g calli. The present study proved that plant regeneration of *B. decumbens* could be accomplished via direct somatic embryogenesis, which involved two stages: initiation of somatic embryos from germinating seeds on MS medium supplemented with 2.0 mgL^{-1} 2,4-D and plantlet regeneration achieved via transferring the somatic embryos onto MS medium without PGRs. Plantlets of *O. sativa* Fujisaka 5 were established via indirect somatic embryogenesis which involved three stages: induction of callus from germinating seeds on MS medium supplemented with 2.0 mgL^{-1} 2,4-D, followed by the production of somatic embryos using MS medium containing 0.5-1.0 mgL^{-1} 2,4-D and kinetin or BAP and NAA and finally plant regeneration by subculturing the somatic embryos onto MS medium without PGRs. These established plant regeneration protocols of *O. sativa* Fujisaka 5 and *B. decumbens* would be useful for future rice improvement research.

Keywords: *Brachiaria decumbens*, cold resistance japonica rice, *Oryza sativa* cv Fujisaka 5, somatic embryogenesis, tropical savanna grass

Abbreviations: 2,4-D: 2,4-Dichlorophenoxy Acetic Acid, BAP: 6-benzylaminipurine, NAA: 1-Naphthaleneacetic Acid, MS: Murashige and Skoog Medium (1962), PGRs: Plant Growth Regulators

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important grain crops providing a global food supply. It is cultivated mostly in Asia (90.6%), Africa (3.5%), the Americas (5.2%), and the balance of 0.7% in Europe and Oceania. Global rice production is projected to reach 582 million tons in 2029, and Asia will contribute much of the world's rice production (OECD/FAO 2020). With the estimated world population of 7.7 billion in 2019 and expected to reach 8.5 billion by 2030 (United Nations 2019), rice production must increase to meet the growing population's demand. Several biotic (diseases, insects) and abiotic (drought, salinity, iron, and aluminum toxicity) stresses are additional causes that reduce rice productivity. Hence, there is a continuous need to develop new rice varieties with higher yield potential and durable resistance to diseases, insects, and abiotic stresses through conventional and biotechnological means.

Rice is the staple food crop in Malaysia. The rice production in Malaysia only fulfills 70% of its rice requirements. The remaining 30% are imported, mainly from Thailand, Vietnam, and Pakistan (Fatah and Cramon-Taubadel 2017). Between 1964 and 2018, Malaysia released 49 rice varieties to increase the production yield with each release of new varieties (Sarena et al. 2019).

Gene transformation and somatic hybridization have been employed as tools for rice improvement. These techniques enable unlimited access to the gene pool by transferring desirable genes. The development of new rice hybrids or rice improvement of different cultivars has been successfully achieved via agrobacterium-mediated transformation, particle bombardment-mediated transformation and protoplast transformation techniques. The Golden rice which produces β-carotene is the successful end-product of these genetic engineering processes (Tan et al. 2017; Page et al. 2019; Poddar et al. 2020)

Hybrid Indica rice is the dominant rice in tropical and subtropical countries. In contrast, the breeding and promotion of hybrid japonica rice are limited, but the demand for japonica rice is continuously increasing in China (Zheng et al. 2020). Cui et al. (2020) reported that the demand and increasing planting of japonica rice varieties have increased for the past decades; hence, it is most likely to be the dominant grain production in China. For the past decade, research on japonica super rice varieties has been actively conducted in Jiangsu Province, China (Wang et al. 2017). The *O. sativa* Fujisaka 5, a cold resistance japonica rice, possessed more panicles and a higher yield than many other rice cultivars. It also matures fast and can be harvested after 111 days of planting, as Dai et al. (1990) reported. *Brachiaria decumbens* Stapf, a tropical and perennial savanna grass, was found to possess desirable genes such as disease and insect resistance, cold and flood tolerance, and high grain quality (Low 2015). These desirable genes of *B. decumbens* could be transferred into the *O. sativa* Fujisaka 5 for future rice improvement via genetic transformation and somatic hybridization techniques. A foreign gene from different plant species other than rice or even bacteria or viruses has been used to create a new variety of transgenic rice with important agronomic traits such as pest resistance, cold and drought tolerance, and resistance to other abiotic and biotic stress via different gene transformation techniques (Low et al. 2018).

Even though there is unlimited information on rice improvement research using various *in vitro* culture techniques, none of these studies were carried out on *O. sativa* Fujisaka 5 and *B. decumbens* except a report on protoplast-derived plants of *O. Sativa* Fujisaka 5 by Ogura et al. (1987). The lack of *in vitro* plant regeneration techniques is one of the main barriers to developing a new rice cultivar. Most of the failures in genetic transformation are mainly due to poor regeneration potential (Rahman et al. 2021). Plant regeneration ability is a prerequisite for a successful transformation process (Mostafiz and Wagiran 2018). The present study was conducted to compare the plant regeneration ability and establish a protocol for *O. sativa* Fujisaka 5 and *B. decumbens* from induced embryogenic callus. Therefore, to the best of the authors' knowledge, the in vitro plant regeneration ability and protocol have not been reported for *O. sativa* Fujisaka 5 and *B. decumbens.*

MATERIALS AND METHODS

Plant materials

Mature seeds of *O. sativa* Fujisaka 5 were obtained from the Malaysian Agriculture Department (MARDI) Gene Bank, Seberang Perai, Penang, Malaysia while the mature seeds of *B. decumbens* were obtained from The International Maize and Wheat Improvement Center (CIMMYT), Mexico.

Establishment of aseptic seed explants

The selected healthy seeds of *O. sativa* Fujisaka 5 were de-husked, washed with mild detergent, and rinsed under running tap water for 30 minutes. The cleansed seeds were then surface sterilized with 70% ethanol for 10 minutes followed by 20% Clorox® (a commercial bleach containing 5.3% sodium hypochlorite, The Clorox Company, Oakland, California, USA) added with a few drops of Teepol (TEEPOL, Malaysia) for 30 minutes and rinsed three times with sterile distilled water (Zainah 2015). The surface-sterilized seeds were inoculated on gelled MS basal medium (Murashige and Skoog 1962) containing 3% sucrose. Furthermore, 10 seeds were used for each sample unit, and 10 sample units were used for the trial. The same procedure was applied to *B. decumbens* seeds. Both seed cultures were kept in a culture room under continuous lighting provided by cool white, fluorescent lights with a light intensity of 30-32.5 $\mu molm^{-2}s^{-1}$ at 25 ± 2°C temperature. The percentage of aseptic seeds established and seed germination were determined after 10 days of culture.

Induction of callus

The aseptic seeds of *O. sativa* Fujisaka 5 and *B. decumbens* were cultured separately on MS medium supplemented with various concentrations (2-10 mgL^{-1}) of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich (M) Sdn. Bhd., Subang Jaya, Malaysia). Next, 10 replicates were used for each medium treatment, and the experiment was repeated 3 (three) times. The cultures were kept in a culture room under continuous lighting provided by cool white fluorescent lights with a light intensity of 30-32.5 $\mu mol\ m^{-2}\ s^{-1}$ at 25 ± 2 °C temperature. After four weeks of culture, the amount of callus induced was determined using a digital scale (Denver Instrument XL-410, Chicago, IL). The best callus induction medium was determined based on the amount of callus produced. The induced calli were transferred to the selected callus proliferation medium (MS + 2 mgL^{-1} 2,4-D) to multiply calli for five subculture cycles (four weeks/cycle). The type of callus produced for each species was then determined.

Induction of embryogenic callus of *Oryza sativa* cv. Fujisaka 5

Effect of MS strength

Approximately 0.5 g of the friable callus of *O. sativa* Fujisaka 5 was inoculated onto different strengths of MS medium (full-strength, ½-strength, ¼-strength, and 1/5-strength) without any plant growth regulator (PGR). Full-strength MS medium contains a full concentration of mineral salts (macro and micronutrients) and organic vitamins of the MS medium while ½-strength, ¼-strength, and 1/5-strength, contain half, a quarter and one-fifth of its concentration respectively. The cultured calli were maintained under continuous light provided by cool white, fluorescent lights with an intensity of 32.5 $\mu Em^{-2}s^{-1}$ at 25 ± 2°C in the culture room. Eight replicates were used for each medium treatment. The experiment was repeated three times. The fresh embryogenic callus mass was determined using a digital weighing scale (Denver Instrument XL-410, Chicago, IL). The proliferation of the embryogenic calli was determined after four weeks of culture based on the growth index according to Godoy-Hernández and Vázquez-Flota (2012).

$$growth\ index = \frac{final\ fresh\ weight(g) - initial\ fresh\ weight(g)}{initial\ fresh\ weight(g)}$$

Effect of 2,4-D and kinetin

Approximately 0.5 g of the friable callus of *O. sativa* Fujisaka 5 were inoculated onto MS medium supplemented with different concentrations of 2,4-D (0, 0.5, and 1.0 mgL^{-1}) and kinetin (0, 0.5 and 1.0 mgL^{-1}) and maintained in the culture room under the similar condition as previous experiments. Eight sample units were used for each combination treatment, and the experiment was repeated three times. The embryogenic callus formation with its growth index and morphology was determined and recorded after four weeks of culture.

Effect of BAP and NAA

Approximately 0.5 g of friable callus of *O. sativa* Fujisaka 5 were inoculated onto MS medium supplemented with different concentrations of 1-Naphthaleneacetic acid (NAA) (0. 0.5 or 1.0 mgL^{-1}) and 6-benzylaminipurine (BAP) (0, 0.5 or 1.0 mgL^{-1}). Eight replicates were used for each medium tested, and the experiment was repeated three times. The growth index of the induced callus for each treatment was calculated, and the morphology and color of the embryogenic callus formed were also observed and recorded after four weeks of culture. All the cultures were incubated in the room under conditions similar to previous experiments.

Plant regeneration ability study

For *B. decumbens,* 0.5 g of the embryogenic callus, derived and proliferated on MS solid medium supplemented with 2.0 mgL^{-1} 2,4-D, was transferred onto PGR-free MS medium. While for *O. sativa cv.* Fujisaka 5, 0.5 g of the embryogenic callus, derived from MS medium supplemented with a combination of 2,4-D and kinetin (0, 0.5, 1.0 mgL^{-1}), and combination of BAP and NAA (0, 0.5, 1.0 mgL^{-1}), were transferred onto PGR-free MS medium. The number of plantlets regenerated was determined after two weeks of cultures. All the cultures were placed in the room under conditions similar to previous experiments.

Histology of *Oryza sativa* cv. Fujisaka 5 somatic embryos

A histology study was done on the green embryogenic callus of *O. sativa* Fujisaka 5 since it can generate normal plantlet formation. The green somatic embryos were removed from the culture medium and fixed immediately in a fixing solution containing 5% formaldehyde, 5% acetic acid and 90% ethanol (FAA) for 24 hours. The fixed tissues were then passed through a series of alcohol solutions, starting with 50% ethanol/tetra butyl alcohol (TBA) (ethanol 95%: absolute TBA: water = 4: 1: 5) and finally treated with absolute ethanol/TBA (ethanol 95%: absolute TBA = 2.5: 7.5). After which the samples were immersed in a mixture solution of TBA and liquid wax with ratio 1: 1 at 60-62 ºC for 24 hours. The solidified wax blocks containing the tissue samples were sliced into 10 µm thickness slices using a rotary microtome (Leica, Germany). The double-stained standard technique was used for the preparation of permanent slides. Each sliced section was placed on a glass slide and treated with a few drops of safranin. Next, a few drops of 95% ethanol were added to remove the excess safranin. Then, it was followed by adding a few drops of Fast Green and 95% ethanol, respectively. Finally, one drop of xylene was added to the section. The sliced section was then covered with a glass cover slip and sealed with Shandon Mount (Shan don, USA) as a mounting agent. The prepared slides were observed under a light microscope and documented (Olympus BX 50, Japan).

Data analysis

The establishment of aseptic seeds and their germination rate for *O. sativa* Fujisaka 5 and *B. decumbens* were compared using the Student t-test. The studies for induction of callus for each species, and the effect of MS strength on induction of embryogenic callus of *O. sativa* Fujisaka were carried out using a completely randomized design. These data were analyzed using one-way ANOVA, and the best callus induction medium or the best MS strength was selected after comparison of the mean using the Tukey´s test at $p \leq 0.05$ with the aid of SPSS version 20.0. The experiments to determine the effect of PGRs on the induction of embryogenic callus of *O. sativa* Fujisaka 5 were carried out using a Complete Randomized Block Design (CRBD). The data collected were then analyzed using two-way ANOVA, and the significant difference of means was compared using Tukey´s test at $p \leq 0.05$ with the aid of SPSS version 20.0. Percentage data were subjected to arcsine transformation before analysis and were converted back to percentages for presentation.

RESULTS AND DISCUSSION

Rice (*O. sativa*) is a monocot plant species comprising Indica and Japonica subspecies. The *O. sativa* Fujisaka 5 is a cold-resistance japonica rice with high-yielding and fast maturing qualities. *B. decumbens*, a tropical and perennial savanna grass, possess disease and insect resistance, cold and flood tolerance genes with high grain quality (Dai et al. 1990; Low 2015). Both *O. sativa* Fujisaka 5 and *B. decumbens* are good candidates for future rice improvement via genetic transformation and somatic hybridization techniques. Therefore, to use these plant species in the crop improvement program, the prerequisite is the establishment of embryogenic calli and plant regeneration protocol in this japonica rice genotype and *B. decumbens.* The establishment of embryogenic calli using mature seeds and both species' plant regeneration abilities have not been studied and reported elsewhere.

Establishment of aseptic seed explants

The aseptic seeds of *O. sativa* Fujisaka 5 (83.3%) and *B. decumbens* (100%) were successfully established by surface-sterilized with 70% ethanol for 10 minutes, followed by 20% Clorox® added with a few drops of Teepol for 30 minutes. After 10 days of culture on MS basal medium, 97.7% of the *O. sativa* Fujisaka 5 seeds germinated while only 40.0% germinated seed were obtained for *B. decumbens* (Table 1). The percentage of seed germinated was significantly different between these two species, but it did indicate the viability of the seeds after the surface sterilization treatment.

Table 1. Percentage of established aseptic seeds and germination rate of *Oryza sativa* Fujisaka 5 and *Brachiaria decumbens*

Plant species	Aseptic seeds (%)	Seed germination (%)
O. sativa Fujisaka 5	83.3 [b]	97.7 [a]
Brachiaria decumbens	100 [a]	40.0 [b]

Mean values within the same column followed by different letters are significantly different at p≤0.05 (Student t-test)

Callus induction

Rice calli have been induced from a wide range of explants, such as mature seeds (Khan et al. 2019), immature embryos (Hoo et al. 2021), anther (Ali et al. 2021), and root pieces (Guo et al. 2018), using different types of culture media and PGRs. The aseptic mature seeds of *O. sativa* Fujisaka 5 and *B. decumbens* showed good response in callus production in biomass when cultured on MS medium supplemented with various concentrations (2-10 mgL^{-1}) of 2,4-D. After four weeks of culture, the amount of callus formed from the mature seeds of *O. sativa* Fijisaka 5 was not dependent on the amount of 2,4-D added into the MS medium. For *B. decumbens*, the amount of callus produced was not significantly different from that of 2,4-D supplemented into the MS medium. Most of the calli formed were mainly from the mesocotyl and radicles of the germinating embryos (Figure 1.A). Even though the initial induction of callus was only 40 ± 2.2% for *O. sativa* Fujisaka 5 and 25.8 ± 1.2% for *B. decumbens*, the induced callus of the two studied species continued to proliferate well when they were subcultured onto MS medium supplemented 2 mgL^{-1} 2,4-D (Table 2). After five subculture cycles (4 weeks/cycle) using the proliferation medium (MS + 2mgL^{-1} 2,4-D), *O. sativa* Fujisaka 5 was mainly made up of friable and non-embryogenic type (Figure 1.B, 1.C). These non-embryogenic calluses were soft, friable, and pale yellow. While for *B. decumbens*, all the calli formed after five subculture cycles on the same proliferation medium were pale purplish green embryogenic type (Figure 1.D). MS Medium supplemented with 2 mgL^{-1} 2,4-D was selected as the best callus proliferation medium because as the concentration of 2,4-D increased, more browning of the induced calli was observed. Pathania and Sandhu (2021) also reported that an increasing concentration of 2,4-D and an increasing frequency of subculturing resulted in a higher incidence of browning in rice calli. However, until today, 2.4-D is still commonly used for various types of rice cultivars (Sathish et al. 2018; Meesook et al. 2020) but has not been reported for callus induction of *O. sativa* Fijisaka 5 and *B. decumbens*.

Induction of embryogenic callus of *O. sativa* Fujisaka 5

Since the induced callus of *O. sativa* Fujisaka 5 remained friable and non-embryogenic even after five subculture cycles (4 weeks/cycle) using MS medium supplemented with 2 mgL^{-1} 2,4-D, other plant growth regulators and different MS strengths were employed to study their effectiveness for inducing the formation of embryogenic callus.

Table 2. Amount of callus induced from the mature seed explants of *Oryza sativa* Fujisaka 5 and *Brachiaria decumbens* after initial four weeks of culture on MS medium supplemented with 2,4-D

Plant species	Concentration of 2,4-D (mgL^{-1})						Callus type
	0	2	4	6	8	10	
	Fresh biomass of callus (mg ± s.e)						
O. sativa Fujisaka 5	No callus growth	40 ± 2.2 a	26 ± 1.6 c	35 ± 2.5 b	33 ± 1.2 bc	36 ± 1.8 b	Friable callus
B. decumbens	No callus growth	25.8 ± 1.2 a	27.1 ± 2.2 a	24.1 ± 5.0 a	23.4 ± 6.5 a	24.5 ± 6.2 a	Embryogenic callus

Note: Mean values within the same row followed by the same letter are not significantly different at p≤0.05 (Tukey´s honest significance test)

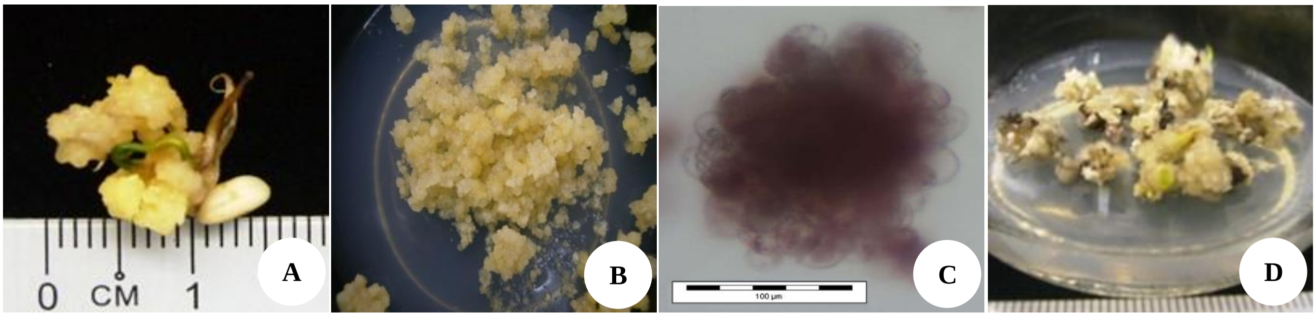

Figure 1. A. Formation of callus at the mesocotyl and radicles of the germinating embryos. B. Friable callus clumps of *Oryza sativa* Fujisaka 5. C. Unorganized callus clumps cells of *O. sativa* Fujisaka 5 view under a microscope (40x magnification). D. Pale purplish green embryogenic callus of *Brachiaria decumbens,* after four weeks of culture on MS medium supplemented with 2 mgL^{-1} 2,4-D

Effect of MS strength

When the friable callus of *O. sativa* Fujisaka 5, derived from MS supplemented with 2 mgL^{-1} 2,4-D, were cultured onto different strengths of MS-salts without any PGR, more than 80% of the callus formed yellow embryogenic callus and the percentage of embryogenic callus formation was not significantly different among the different MS strength. When the MS-salts decreased, 9.5-19.2% of the callus turned brown and remained friable. Only 3.2% of the calli remained friable when full MS-salts were used. The Growth Index (GI) of the embryogenic callus was also reduced as the MS strength decreased (Table 3). Hence full-strength MS medium was used for the induction of embryogenic callus for the subsequent studies. Several different basal media such as MS (Murashige and Skoog 1962), LS (Linsmaier and Skoog 1965) and N6 (Chu 1978) have been used in rice tissue culture. Vennapusa et al. (2015) have applied these three basal media for callus induction and regeneration for a drought-tolerant rice indica genotype and found that the highest embryogenic callus induction frequency (91.3%) was established using LS basal medium supplemented with 2,4-D. However, until today many researchers still found full MS with a combination of different PGRs are suitable for induction of embryogenic callus in various rice cultivars, such as Himalaya rice (Noor et al. 2022), indica and japonica rice subspecies (Carsono et al. 2021), and Malaysia indica rice (Ng at al. 2019).

Effect of 2,4-D and kinetin

After four weeks of culture on MS medium containing 2,4-D or kinetin or combining 2,4-D and kinetin, three colored types of embryogenic calli were formed. The embryogenic calli formed were mostly the yellowish type (85.8-92.6%), and the remainder were of the greenish and brownish type of embryogenic callus. There was no significant difference in the percentage formation of yellowish embryogenic callus type in all tested concentrations 2,4-D and kinetin combination. The combination of 2,4-D and kinetin supplemented into the MS medium greatly affected the formation of the greenish and brownish type of embryogenic callus. The growth of the embryogenic callus based on growth index was high when they were cultured on MS medium without 2,4-D and kinetin, or only with the presence of 2,4-D (0.5mgL^{-1}, 1.0 mgL^{-1}) or with the presence of both 2,4-D (0.5mgL^{-1}, 1.0 mgL^{-1}) and kinetin (1.0 mgL^{-1}) (Table 4). However, only the green and yellowish type of embryogenic callus could grow if they were subcultured onto the selected embryogenic callus maintenance medium (MS + 0.5 mgL^{-1} 2,4-D + 1.0 mgL^{-1} kinetin) and remained healthy at the embryogenic state. In contrast, the brownish callus died off after four weeks of culture. Paramasivam and Harikrishna (2020) also reported the addition of 2,4-D (2.5 mgL^{-1}) and kinetin (0.1 mgL^{-1}) into the MS medium induced embryogenic callus in Malaysian wild red rice. In contrast, Paul and Roychoudhury (2019) induced embryogenic calli in indigenous aromatic indica rice varieties using MS medium containing only 2,4-D (3 mgL^{-1}). But both authors have not reported the effect of PGRs on browning of the embryogenic calli as found in our present study. Similarly, in our present study, only 2,4-D (2mgL^{-1}) supplementation was required to induce the embryogenic callus of *B. decumbens*.

Table 3. Effects of different MS strengths without PGRs on induction of embryogenic callus of *Oryza sativa cv. Fujisaka 5*.

MS strength	**Growth Index (GI) (mean ± s.e)**	**Percentage formation and embryogenic callus Type**	
		Yellowish embryogenic (%) (mean ± s.e)	**Brownish friable (%) (mean ± s.e)**
Full-strength MS	4.3 ± 1.8 ^{a}	96.8 ± 3.5 ^{a}	3.2 ± 3.5 ^{b}
Half-strength MS	3.4 ± 1.2 ^{ab}	84.0 ± 14.1^{a}	16.0 ± 14.1 ^{a}
1/4-strength MS	3.1 ± 0.8 ^{ab}	80.8 ± 16.9 ^{a}	19.2 ± 16.9 ^{a}
1/5-strength MS	2.4 ± 0.8 ^{b}	90.5 ± 6.9 ^{a}	9.5 ± 6.9 ^{ab}

Note: Mean values within the same column followed by the same letter are not significantly different at p≤0.05 (Tukey´s honest significance test)

Table 4. Effects of 2,4-D and kinetin supplemented into MS medium on formation of Embryogenic callus of *Oryza sativa* cv. Fujisaka 5

MS medium supplemented with		**Growth Index**	**Type of callus (% mean ± se)**		
2,4-D (mgL^{-1})	**Kinetin (mgL^{-1})**	**(GI) ± se**	**Greenish**	**Yellowish**	**Brownish**
0	0	3.5 ± 0.4^{a}	2.4 ± 0.2^{b}	85.8 ± 5.9^{a}	11.7 ± 2.5^{a}
0.5	0	3.3 ± 0.5^{a}	1.8 ± 0.4^{c}	91.6 ± 2.9^{a}	6.6 ± 1.0^{ab}
1.0	0	3.2 ± 0.6^{a}	0.4 ± 0.4^{d}	95.6 ± 2.1^{a}	4.0 ± 0.8^{c}
0	0.5	1.0 ± 0.3^{c}	0.8 ± 0.6^{d}	93.8 ± 2.6^{a}	5.4 ± 1.2^{b}
0.5	0.5	2.2 ± 0.6^{b}	2.3 ± 0.4^{bc}	89.1 ± 3.1^{a}	8.7 ± 1.6^{a}
1.0	0.5	2.1 ± 0.6^{b}	4.5 ± 0.9^{a}	93.2 ± 2.1^{a}	2.3 ± 0.9^{d}
0	1.0	1.8 ± 0.5^{b}	1.4 ± 0.2^{cd}	96.2 ± 1.2^{a}	2.4 ± 0.8^{d}
0.5	1.0	3.1 ± 0.6^{ab}	4.3 ± 0.9^{a}	91.5 ± 3.3^{a}	4.2 ± 1.7^{c}
1.0	1.0	2.7 ± 0.6^{ab}	0.2 ± 0.2^{d}	92.6 ± 4.5^{a}	7.2 ± 1.5^{a}

Note: Mean values within the same column followed by the same letter are not significantly different at p≤0.05 (Tukey´s honest significance test)

Table 5. Effects of NAA and BAP supplemented into MS medium on *Oryza sativa* cv Fujisaka 5 embryogenic callus formation

MS Medium supplemented with		Growth Index	Type of callus (% mean ± se)		
BAP (mgL^{-1})	NAA (mgL^{-1})	(GI) ± se	Greenish	Yellowish	Brownish
0	0	5.2 ± 0.4 ^{a}	0 ^{a}	100.0± 0.0 ^{a}	0 ^{a}
0.5	0	3.9 ± 0.4 ^{ab}	0.1 ± 0.1 ^{a}	97.2 ± 1.5 ^{a}	2.7 ± 1.5 ^{a}
1.0	0	3.9 ± 0.4 ^{ab}	0.7 ± 0.5 ^{a}	98.2 ± 0.7 ^{a}	1.1 ± 0.7 ^{a}
0	0.5	4.8 ± 0.5 ^{ab}	0 ^{a}	99.2 ± 0.4 ^{a}	0.8 ± 0.4 ^{a}
0.5	0.5	3.4 ± 0.2 ^{b}	0 ^{a}	98.3 ± 1.0 ^{a}	1.7 ± 1.0 ^{a}
1.0	0.5	3.4 ± 0.3 ^{b}	0.6 ± 0.6 ^{a}	98.6 ± 0.6 ^{a}	0.8 ± 0.3 ^{a}
0	1.0	2.4 ± 0.5 ^{b}	0.8 ± 0.6 ^{a}	96.8 ± 1.7 ^{a}	2.4 ± 1.6 ^{a}
0.5	1.0	3.2 ± 0.4 ^{b}	0 ^{a}	100.0 ± 0.0 ^{a}	0 ^{a}
1.0	1.0	2.7 ± 0.4 ^{b}	0.2 ± 0.2 ^{a}	99.3 ± 0.5 ^{a}	0.4 ± 0.4 ^{a}

Note: Mean values within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey´s honest significance test)

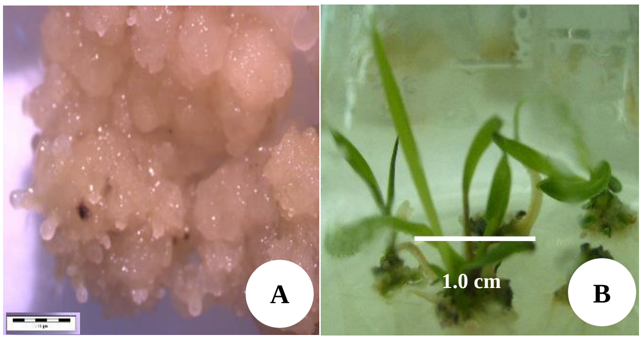

Figure 2. A. Somatic embryos of *B. decumbens* cultured on MS medium supplemented with 2 mgL^{-1} 2,4-D. B. Plantlet regeneration of *B. decumbens* after two weeks cultured of embryogenic callus on PGR-free MS medium

Effect of BAP and NAA

Most of the friable calli of *O. sativa* Fujisaka 5 formed embryogenic callus (96%-100%) and remained yellowish after four weeks of culture on MS medium without BAP or NAA or MS medium supplemented with 0.5 mgL^{-1} to 1.0 mgL^{-1} BAP and/or NAA. A small amount of the embryogenic callus formed were of the greenish or brownish type. A high growth index was observed in embryogenic callus cultured on MS medium without plant growth regulators or MS medium supplemented with only BAP or NAA (Table 5). Not many researchers reported the effectiveness of BAP and NAA supplemented into MS medium for induction of rice embryogenic callus. However, these two PGRs were efficient for embryogenic callus induction in other plant species such as *Nicotiana tabacum* cv. TAPM26 (Moh Hussein et al. 2020) and *Nigella damascene* L. (Klimek-Chodacka et al. 2020).

Plant regeneration ability

Brachiaria decumbens

The plant regeneration of *B. decumbens* was straight forward because all the embryogenic callus with greenish somatic embryos derived from MS + 2.0 mgL^{-1} 2,4-D germinated into plantlet after two weeks of transferring onto PGR-free MS medium (Figure 2.A-2.B). An average of 4 to 5 plantlets could be obtained from 0.5g of the inoculated embryogenic callus.

Oryza sativa cv. Fujisaka 5

The embryogenic callus of *O. sativa* Fujisaka 5 induced using different MS-salts strength or MS medium without any PGRs could not regenerate into plantlets. After three weeks of culture, the embryogenic callus derived from the lower MS Strength turned brown from the original yellow callus. This might be due to the stress caused by the initial culture in a PGR-free medium and low salt concentration. The lowest MS-strength (1/5 MS-strength) caused the highest percentage of brown callus (up to 90%). The embryogenic callus inoculated on full MS-strength medium exhibited 2% of green embryos but failed to regenerate into plantlets. These results indicated that the embryogenic callus of *O. sativa* Fujisaka 5 derived from any strength of MS medium without any PGRs could not develop into plantlets when transferred directly onto PGR-free MS medium.

Three colored types of embryogenic callus, yellow, green, and brown, were induced when cultured on MS medium supplemented with a combination of 2,4-D and kinetin or MS medium supplemented with a combination of BAP and NAA (Figure 3.A). A high percentage of the embryogenic callus formed was the yellowish type that could not germinate to form plantlets when transferred onto a PGR-free MS medium. Only the greenish type of embryogenic callus, mostly at the heart or torpedo stage of embryos (Figure 3.B), were generated into plantlets after two weeks cultured on the PGR-free MS medium. Via observation, it was found that the plant regeneration ability corresponded well with the amount of greenish type of embryogenic callus formation. These green somatic embryos gave rise to normal plantlets when sub-cultured onto fresh PGR-free MS medium after two weeks (Figure 3.C). MS medium + 1.0 mgL^{-1} 2,4-D + 0.5 mgL^{-1} kinetin or MS medium + 0.5 mgL^{-1} 2,4-D + 1.0 mgL^{-1} kinetin could induce the formation of 4.3 to 4.5% of green embryogenic callus (Table 4) and these green embryogenic calli continue to proliferate well when they were subcultured on these proliferative media. Continuous subculturing on these proliferative media produced the green embryogenic calli which could be used to regenerate plantlets. An average of two to three plantlets could be developed from 0.5 g of the inoculated embryogenic callus after two weeks transferred onto a PGR-free MS medium.

This present study showed that the low regeneration ability of *O. sativa* Fujisaka 5 was largely due to the problem of the low germination rate of the induced somatic embryos. Several factors, including plant genotypes, explant type, plant growth regulators, and culture conditions, could subsequently affect somatic embryos germination and plant regeneration (Ghobeishavi et al. 2015). Cellular differentiation and accumulation of reserve substances are also key factors for successfully converting somatic embryos into plantlets (Mishra et al. 2012). Mature seed explants were found to have a low frequency of plant regeneration from somatic embryos to plant regeneration (Verma et al. 2011).

Histology of *Oryza sativa* cv. Fujisaka 5 somatic embryos

Histological study of *O. sativa* Fujisaka 5 somatic embryos derived from the induced embryogenic callus (Figure 4.A) showed primordial leaves and meristematic shoot formation with plant regeneration after two weeks cultured on the PGR-free MS medium. The differentiated primordial leaves and shoot comprised small isodiametric cells with prominent nuclei and dense cytoplasm (Figure 4.B). After four weeks of culture, a complete plantlet was formed with well-developed roots.

Results of our study showed that *in vitro* plant regeneration of *B. decumbens* and *O. sativa* Fujisaka 5 could be established via somatic embryogenesis technique but with a slightly different protocol. Plant regeneration of *B. decumbens* could be accomplished via direct somatic embryogenesis, which involved two stages, initiation of somatic embryos on MS medium supplemented with 2 mgL^{-1} 2,4-D and plantlet regeneration after transferring the somatic embryos onto PGR-free MS medium. For *O. sativa* Fujisaka 5, plantlet regeneration was established via indirect somatic embryogenesis process. It involved three stages, formation of non-embryogenic callus on MS medium supplemented with 2 mgL^{-1} 2,4-D, followed by induction of embryogenic callus and somatic embryos using MS medium + 1.0 mg/L 2,4-D + 0.5 mg/L kinetin or MS medium + 0.5 mg/L 2,4-D + 1.0 mg/L kinetin. Finally, plant regeneration could be achieved by transferring these embryogenic calli and somatic embryos onto a PGR-free MS medium.

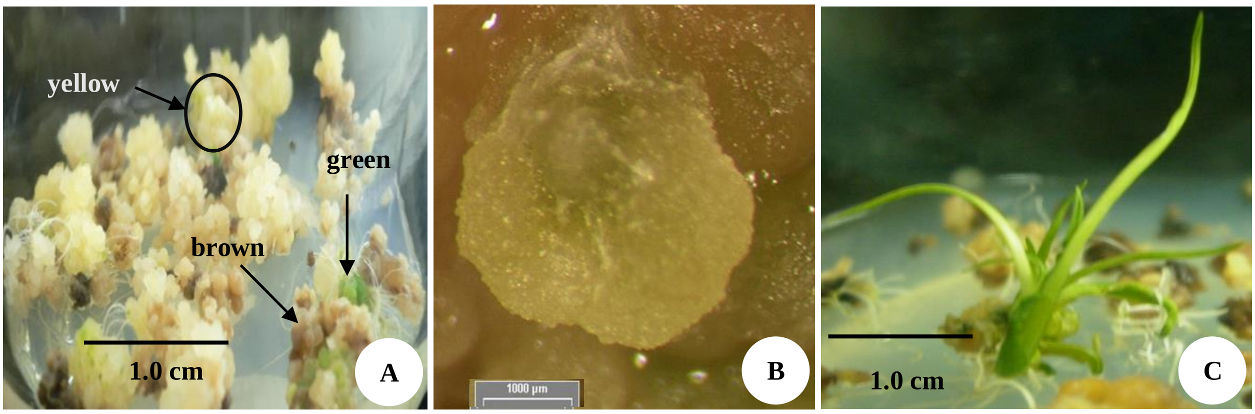

Figure 3. A. Mixture of yellow, green and brown embryogenic callus with some friable callus and somatic embryos of *Oryza sativa* Fujisaka 5 established on MS medium + 1.0 mgL^{-1} 2,4-D + 0.5 mgL^{-1} kinetin. B. Heart/torpedo stage of somatic embryos existing in green embryogenic callus of culture (A). C. Plantlet regeneration of *O. sativa* Fujisaka 5 after two weeks cultured of (A) on PGR-free MS medium.

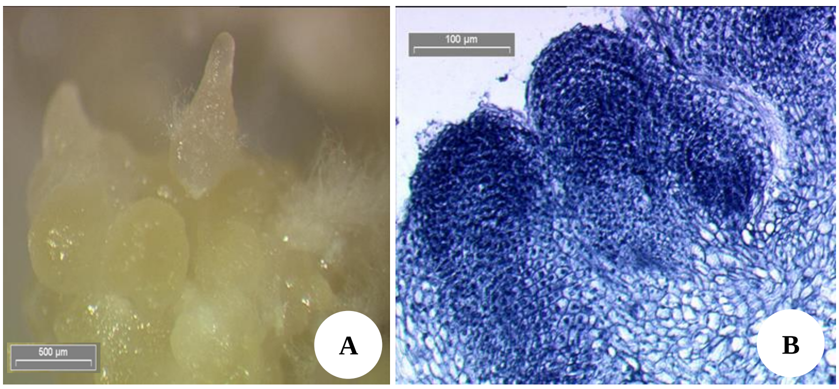

Figure 4. A. Somatic embryos of globular and Torpedo stage developed from the induced embryogenic callus. B. Formation of primordial leaves and meristematic shoot of germinated *Oryza sativa* Fujisaka 5 somatic embryo

ACKNOWLEDGEMENTS

The authors wish to thank the School of Biological Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia for facilities and resources for research. This study was carried out without any funding from any funding agency. Appreciation is also given to the Institute of Higher Studies, USM, Penang, Malaysia for the post-graduate fellowship to the first author. The authors declare that they have no conflict of interest. This article does not contain any studies using human participants or animals.

REFERENCES

Ali J, Nicolas KLC, Akther S, Torabi A, Ebadi AA, Marfori-Nazarea CM, Mahender A. 2021. Improved anther culture media for enhanced callus formation and plant regeneration in rice (*Oryza sativa* L.). Plants 10 (5): 1-16. DOI: 10.3390/plants10050839.

Carsono N, Juwendah E, Liberty, Sari S, Damayanti F, Rachmadi M. 2021. Optimize 2,4-D concentration and callus induction time enhance callus proliferation and plant regeneration of three rice genotypes. Biodiversitas 22 (7): 2555-2560. DOI: 10.13057/biodiv/d220702.

Chu CC. 1978. The N6 medium and its applications to anther culture of cereal crops. Proceedings of Symposium on Plant Tissue Culture, Science Press, Peking, China, 25-30 May 1978.

Cui Y, Zhu MM, XU ZJ, CHEN WF. 2020. The breeding of japonica rice in northern China: An 11-year study (2006-2016). J Integr Agric 19 (8): 1941-1946. DOI: 10.1016/S2095-3119 (19)62799-1.

Dai Q, Vergara BS, Visperas RM. 1990. Amelioration of cold injury in rice (*Oriza sativa* L.): Improving root oxidizing activity by plant growth regulators. Philipp J Crop Sci 15 (1): 49-54.

Fatah FA, Cramon-Taubadel S. 2017. Profitability and competitiveness of rice farming in malaysia: A policy analysis matrix. AJARD 14 (2): 31-47. DOI: 10.22004/ag.econ.265764

Ghobeishavi H, Uliaie ED, Alavikia SS, Valizadeh M. 2015. Study of factors influencing somatic embryogenesis in rice (*Oryza sativa* L.). Intl J Adv Biol Biom Res 3 (1): 43-50.

Godoy-Hernández G, Vázquez-Flota FA. 2012. Growth measurements: Estimation of cell division and cell expansion. Methods Mol Biol 877: 41-48. DOI: 10.1007/978-1-61779-818-4_4.

Guo F, Zhang H, Liu W, Hu X, Han N, Qian Q, Xu L, Bian H. 2018. Callus initiation from root explants employs different strategies in rice and arabidopsis. PCP 59 (9): 1782-1789. DOI: 10.1093/pcp/pcy095.

Hoo KY, Lee PC, Latip MA, Saruyau N, Aziz ZA. 2021. Callus formation and plant regeneration from immature embryos of pigmented upland rice (*Oryza sativa* cv. Tadong). J Technol Sci Eng 83 (4): 91-100. DOI: 10/11113/jurnalteknologi.v83.14518.

Khan MdNM, Islam MdM, Islam MdS, Uddin MdI. 2019. Studies on in vitro response to callus induction and gene transfer technique of five high yielding indica rice varieties. J Sci Agric 3: 41-45. DOI: 10.25081/jsa.2019.v3.5465.

Klimek-Chodacka M, Kadluczka D, Lukasiewicz A, Malec-Pala A, Baranski R, Grzebelus E. 2020. Effective callus induction and plant regeneration in callus and protoplast cultures of *Nigella damascena* L. PCTOC 143: 693-707. DOI: 10.1007/s11240-020-01953-9.

Linsmaier EM, Skoog F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18: 100-127. DOI: 10.1111/j.1399-3054.1965.tb06874.x.

Low LY, Yang SK, Kok DXA, Ong-Abdullah J, Tan NP, Lai KS. 2018. Transgenic Plants: Gene Constructs, Vector and Transformation Method. New Visions in Plant Science. Intech, London. DOI: 10.5772/intechopen.79369.

Low SG. 2015. Signal grass (*Brachiaria decumbens*) toxicity in grazing ruminants. Agriculture 5: 971-990. DOI: 10.3390/agriculture5040971.

Meesook K, Pongtongkam P, Poeaim A. 2020. Duration for callus propagation of indica rice (*Oryza sativa* L.) cultivar Sangyod in suspension culture. IJAT 16 (1): 77-86.

Mishra S, Sanyal I, Amla DV. 2012. Changes in protein pattern during different developmental stages of somatic embryos in chickpea. Biol Plant 56 (4): 613-619. DOI: 10.1007/s10535-012-0124-0.

Moh Hussein N, Huyop F, Kaya Y. 2020. An easy and reliable method for establishment and maintenance of tissue cultures of *Nicotiana tabacum* cv. TAPM 26. Intl J Sci Lett 2 (2): 62-71. DOI: 10.38058/ijsl.764947.

Mostafiz S, Wagiran A. 2018. Efficient callus induction and regeneration in selected indica rice. Agronomy 8 (5): 1-18. DOI: 10.3390/agronomy8050077.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.

Ng JM, Mostafiz S, Johon NS, Zulkifli NSA, Wagiran A. 2019. Combination of plant growth regulators, maltose, and partial desiccation treatment enhances somatic embryogenesis in selected Malaysian rice cultivar. Plants 8 (144): 1-15. DOI: 10.3390/plants8060144.

Noor W, Lone R, Kamili AN, Husaini AM. 2022. Callus induction and regeneration in high altitude Himalayan rice genotype SR4 via seed explant. Biotechnol Rep 36 (2022): 1-8. DOI: 10.1016/j.btre.2022.e00762.

OECD/FAO 2020. OECD-FAO Agricultural Outlook 2020-2029. FAO, Rome/OECD Publishing, Paris. DOI: 10.1787/1112c23b-en.

Ogura H, Kyozuka J, Hayashi Y., Koba T., Shimamoto K. 1987. Field performance and cytology of protoplast-derived rice (*Oryza sativa*): high yield and low degree of variation of four japonica cultivars. Theoret Appl Genetics 74: 670-676. DOI: 10.1007/BF00288869.

Page MT, Parry MAJ, Carmo-Silva E. 2019. A high-throughput transient expression system for rice. Plant Cell Environ 42 (7): 2057-2064. DOI: 10.1111/pce.13542.

Paramasivam S, Harikrishna JA. 2020. Effect of culture media and conditions on callus induction and plant regeneration of Malaysian wild rice *Oryza rufipogon* IRGC105491. Res J Biotechnol 15 (3): 120-127.

Pathania S, Sandhu JS. 2021. Effect of 2,4-d on embryogenic callus induction and evaluation of g418 on growth inhibition in rice calli. Agric Res J 58 (1): 18-22. DOI: 10.5958/2395-146X.2021.00002.8.

Paul S, Roychoudhury A. 2019. Comparative analyses of regeneration potentiality of eight indigenous aromatic indica rice (*Oryza sativa* L.) varieties. Intl J Biol Sci 6 (1): 55-64. DOI: 10.26438/ijsrbs/v6i1.5564.

Poddar S, Tanaka J, Cate JHD, Staskwicz B, Cho MJ. 2020. Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays. Plant Methods 16 (1): 1-11. DOI: 10.1186/s13007-020-00692-4.

Rahman MT, Hasanuzzaman M, Islam MM, Mondal MTR, Islam MS, Saha NR. 2021. Optimization for in vitro regeneration in four local cultivars of indica rice. Asian J Agric Biol 2021 (4): 1-9. DOI: 10.35495/ajab.2021.01.045.

Sarena CO, Shaharudin A, Tumin SA. 2019. The Status of the Paddy and Rice Industry in Malaysia. Khazanah Research Institute, Kuala Lumpur.

Sathish S, Venkatesh R, Safia N, Sathishkumar R. 2018. Studies on growth dynamics of embryogenic cell suspension cultures of commercially important Indica rice cultivars ASD16 and Pusa basmati. 3 Biotech 8 (194): 1-9. DOI: 10.1007/s13205-018-1213-3.

Tan LW, Rahman ZA, Goh HH, Hwang DJ, Ismail I, Zainal Z. 2017. Production of transgenic rice (indica cv. MR219) overexpressing ABP57 gene through Agrobacterium-mediated transformation. Sains Malays 46 (5): 703-711. DOI: 10.17576/jsm-2017-4605-04.

United Nations. 2019. World Population Prospects 2019. Department of Economic and Social Affairs, New York City.

Vennapusa AR, Vemanna RS, Rajashekar RBH, Babitha KC, Kiranmai K, Nareshkumar A, Sudhakar C. 2015. An efficient callus induction and regeneration protocol for a drought tolerant rice indica genotype AC39020. J Plant Sci 3 (5): 248-254. DOI: 10.11648/j.jps.20150305.11.

Verma D, Joshi R, Shukla A, Kumar P. 2011. Protocol for in vitro somatic embryogenesis and regeneration of rice (*Oryza sativa* L.). Indian J Exp Biol 49 (12): 958-963.

Wang CL, Zhang YD, Zhu Z, Chen T, Zhao QY, Zhong WG, Yang J, Yao S, Zhou LH, Zhao L, Li YS. 2017. Research progress on the breeding of japonica super rice varieties in Jiangsu Province, China. J Integr Agric 16 (5): 992-999. DOI: 10.1016/S2095-3119 (16)61580-0.

Zainah D. 2015. Protoplast Culture of *Oryza sativa* L. and *Brachiaria decumbens*. [Dissertation]. Universiti Sains Malaysia, Penang, Malaysia.

Zheng W, Ma Z, Zhao M, Xiao M, Zhao J, Wang C, Gao H, Bai Y, Wang H, Sui G. 2020. Research and development strategies for hybrid japonica rice. Rice 13 (1): 1-22. DOI: 10.1186/s12284-020-00398-0.

CELL BIOLOGY & DEVELOPMENT
Volume 7, Number 1, June 2023 E-ISSN: 2580-4499
Pages: 28-34 DOI: 10.13057/cellbioldev/v070104

Effects of green-synthesized silver nanoparticles from *Azadirachta indica* on growth performance and liver function parameters in male albino rats

KAYODE ADEBISI AROWORA, KENNETH CHINEKWU UGWUOKE, MOSES ADONDUA ABAH, BONAVENTURE CHINECHEREM UGWOKE
Department of Biochemistry, Faculty of Pure and Applied Sciences, Federal University Wukari. Katsina Ala Rd, Wukari, Taraba State, Nigeria. Tel.: +234-8030000000, ♥email: aroworak2002@gmail.com

Manuscript received: 22 September 2022. Revision accepted: 30 June 2023.

Abstract. *Arowora KA, Ugwuoke KC, Abah MA, Ugwoke BC. 2023. Effects of green-synthesized silver nanoparticles from* Azadirachta indica *on growth performance and liver function parameters in male albino rats. Cell Biol Dev 7: 28-34.* The effects of green-synthesized silver nanoparticles (AgNPs) from *Azadirachta indica* A.Juss. aqueous leaf extract on growth performance and liver function parameters in male albino rats were evaluated. Synthesis of AgNPs from $AgNO_3$ and plant extract was achieved using standard methods, while the characteristic peak of the synthesized AgNPs was determined using a UV-VIS spectrophotometer at a resolution of 1 nM. The crystal structure of AgNPs was determined using the technique of Fourier Transform Infrared Spectroscopy (FTIR). Growth performance study was carried out using the AOAC method. A total of 16 male albino rats weighing between 120 and 180 g were randomly allotted into four experimental groups having four rats each. 100, 250, and 500 mg/kg AgNPs were administered daily to Groups 2, 3, and 4 respectively for 14 days, while Group 1 served as the control. At the end of the administration, rats were sacrificed under anaesthetization, and serum samples were collected for analysis. The result revealed that the absorption characteristic peak of the ultraviolet-visible spectrum of the silver nanoparticles synthesized was 450 nM. It was also observed that the growth performance of the experimental animals gave no significant difference in average weight gain, feed intake, and feed efficiency ratio. The feed conversion ratio of Group 4 (6.94) is significantly higher than all the groups. However, Groups 2 (4.56) and 3 (4.74) were found to be non-significant ($P>0.05$), and significantly higher than the Control Group (3.75). Synthesized silver nanoparticles from *A. indica* affected the level of some selected biochemical parameters such as ALT, ALP, TP AST, and Albumin in a non-significant way. This study found no evidence of hazardous effects from silver nanoparticles, which could be attributed to the minimal dosage of AgNPs used or the source of the nanoparticles.

Keywords: *Azadirachta indica*, growth performance, liver function tests, silver nanoparticles

INTRODUCTION

Silver nanoparticles (AgNPs) are extremely small silver atoms with sizes ranging from 1 nM to 100 nM (Graf et al. 2003). Owing to their antibacterial qualities, silver NPs have been used extensively in the food and medical industries (Pulit et al. 2015). Sahayaraj and Rajesh (2011) reported that several public places in China utilize AgNPs as an antimicrobial agents, and it has been used greatly to lessen the infections of implanting surgical catheters in the course of surgery. It is no longer news that many researchers have put forward many biological activities; these AgNPs possess, ranging from anti-angiogenic, anti-fungal, anti-permeability, and to anti-inflammatory activities (Gurunathan et al.2009; Kalishwaralal et al. 2009; Sheikpranbabu et al. 2009).

In recent years, the biosynthesis of AgNPs has been at the forefront of contemporary nanotechnology (Gou et al. 2015; Parang and Moghadamnia 2018), and it has been identified that several biological agents, including microbes and plant extracts, have the capability of reducing silver ions to AgNP (Kahrilas et al. 2014; Dewi et al. 2023). According to several investigations, oxidative stress that causes lipid peroxidation and even apoptosis or necrosis which emanates from an imbalance between the generation of Reactive Oxygen Species (ROS) and antioxidant defenses, is the mechanism that significantly contributes to the cytotoxic effects of nanosilver (Kim and Ryu 2013; Völker et al. 2013). Reactive oxygen species production and alterations in enzyme activity linked to antioxidant defense systems were revealed to be induced by AgNPs (Dziendzikowska et al. 2012).

The likelihood of these nanoparticles becoming harmful rises with their widespread use. According to several scientific and medical studies, nanoparticles can enter the body through the skin, lungs, and gastrointestinal tract and cause negative health effects (Wijnhoven et al. 2009). Several consumers' items often contain silver nanoparticles because of their antibacterial and anti-fungal characteristics. These Ag NP-containing items include fabric (Kulthong et al. 2010), aerosolized such as nasal sprays or air sanitizers (Chao et al. 2011), wound dressings solvent (Silver et al. 2006), kinds of toothpaste, and colloidal silver suspensions (Nowack et al. 2011), has direct or indirect exposure to humans.

The liver, being one of the most crucial organs, is the main target for any routes of exposure involving bloodstream translocation, and prior studies have demonstrated a significant accumulation of NPs inside the liver following injection (Hirn et al. 2011), retention of particles in the liver after ingestion (Schleh et al. 2012), and liver affected following inhalation. To ensure customers' safety, it is necessary to screen for AgNPs' potential toxicity. Therefore, this work is aimed at determining the effect of synthesized silver nanoparticles from a plant origin (*Azadirachta indica* A.Juss.) on the performance and liver function tests in male albino rats.

An expanding field of study is the green production of nanoparticles, which is a well-known substitute for conventional techniques. This method utilizes plant extracts for the biosynthesis of nanoparticles. The advantages of green syntheses over chemical and physical approaches include environmental friendliness, cost-effectiveness, and ease of scaling up for large-scale nanoparticle production. In addition, there is no need to utilize harmful compounds or high temperatures, pressures, or energies (David et al. 2014). Taking into account the enormous potentiality of plants as a source, this work synthesized AgNPs using *A. indica* aqueous leaf extract.

The *A. indica*, usually called neem, is a species from a family called Meliaceae, which was used to bioconvert silver ions into nanoparticles. This plant is widely distributed in India, and from ancient times, all parts of the tree have been employed as natural remedies for a variety of human maladies, including viral infections. The plant leaves contain β-sitosterol (a flavonoid that is polyphenolic), and quercetin that possess anti-fungal and antibacterial activities (Srivastava et al. 2020). According to reports, neem leaves and their constituents have immuno-modulatory, anti-inflammatory, anti-hyperglycemic, ulcer-healing, antioxidant, anticarcinogenic, anti-mutagenic, antimalarial, antibacterial, anti-fungal, and antiviral properties (Girish and Shankara 2008).

MATERIALS AND METHODS

Chemicals and plant material collection

All the reagents purchased were of analytical grade and used without any further purification. Silver nitrate ($AgNO_3$) was purchased from Merck with a ≥99.5% purity. Neem leaves (*A. indica*) were collected from Wukari environs into a polyethylene zipper bag and transported to the Biochemistry Research Laboratory, Federal University Wukari, Taraba State, Nigeria.

Preparation of plant sample

The extraction of the plant sample was done using the method described by David et al. (2014). The *A. indica* leaf extract was prepared with distilled water. It was thoroughly washed with tap water and then with distilled water and cut into small pieces. The chopped leaves were then ground using mortar and pestle and 10g was weighed and heated in 100 mL of distilled water for 30 minutes at 60°C. The leaf broth was then cooled and filtered using Whatman No. 1 paper.

Synthesis of silver nanoparticles and its characterization

The biosynthesis of Silver Nanoparticles was done using plant materials as described by Sahayaraj and Rajesh (2011). A stock solution of 1 mM silver nitrate ($AgNO_3$) was prepared with distilled water.Ten (10) mL of *A. indica* leaf extract was added to 200 mL of 1 mM $AgNO_3$ solution placed on a hot plate with mild stirring. After heating the solution at 60°C for 30 minutes, there was a change in colour from dark green to yellow which indicates the formation of silver nanoparticles. The characteristic peaks were determined using a UV-VIS spectrophotometer at a resolution of 1 nM, by periodically scanning the optical absorbance between a range of 300-600 nM to investigate the reduction rate of silver ions by the leaf extract. The crystals of green synthesized silver nanoparticles produced was subjected to FTIR spectroscopy for its characterization.

Experimental animals

Male rats of Wistar strain weighing between 120 g and 180 g were obtained from National Veterinary Research Institute Vom, Plateau state, Nigeria. Animals were housed in a hygienic environment and allowed to acclimatize for a week before the commencement of treatments. Animals were fed with commercial rat chows and given potable water *ad libitum.*

Animal groupings and treatments

Animals were randomly assigned into four experimental Groups 1-4 of four rats per group. Group 1 served as control and received potable water daily. Groups 2, 3, and 4 were daily administered with 100, 250, and 500 mg/kg AgNPs respectively for 14 days. All treatments were orally administered with the aid of a cannula.

Liver function tests

At the end of two weeks of administration, rats were sacrificed under anaesthetization in slight chloroform. Blood samples were obtained by cardiac puncture into plain bottles. The blood samples were allowed to coagulate and then centrifuged for 10 min at 3000 rpm. Serum was obtained by aspiration of the clear yellowish liquid. Estimation of liver function tests, such as AST (Aspartate Transaminase), ALT (Alanine Transaminase), Alkaline phosphatase (ALP), Total Protein (TP), and Albumin (ALB) were carried out using serum with the aid of biochemical kits.

Growth performance study

The growth performance was studied using the method of AOAC (2000), Feed Intake is the sum of the quantity of feed consumed by the rats. The average weekly weight gain, feed efficiency ratio, and feed conversion ratio were calculated using the following formulas:

$$Feed\ Efficiency\ Ratio\ (FER) = \frac{Body\ weight\ gain}{feed\ intake} \quad (1)$$

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{feed\ intake}{Body\ weight\ gain} \quad (2)$$

Weight gained by the rats is an increase in body weight. In each group,

$$Body\ Weight\ Gain\ (BWG) = Average\ final\ live\ weight - initial\ live\ weight \quad (3)$$

Statistical analysis

All the analyses were carried out in duplicates in completely randomized design. The data were subjected to analysis of variance using Statistical Package for Social Science (SPSS) software. Means that were significantly different were separated by the Least Significant Difference (LSD) test. Significance was accepted at $P<0.05$.

RESULTS AND DISCUSSION

Synthesis of AgNPs from $AgNO_3$ and *Azadirachta indica* leaf extract

The biosynthesis of Silver Nanoparticles was done using plant materials as described by Sahayaraj and Rajesh (2011). After heating the solution at 60°C for 30 minutes, there was a change in colourfrom dark green to yellow which indicates the formation of silver nanoparticles (Figure 1).

Spectrophotometric evaluation of $AgNO_3$ reduction

In this study, silver nanoparticles were synthesized from plant material. The absorption characteristic peak of the ultraviolet-visible spectrum of the silver nanoparticles synthesized wasobserved at 445 nM (Figure 2).

FTIR spectroscopy characterization of green synthesized silver nanoparticles

The crystals of green synthesized silver nanoparticles produced was subjected to FTIR spectroscopy for its characterization. The changes observed in the reaction converting $AgNO_3$ to AgNPs using FTIR analysis is represented below (Figure 3).

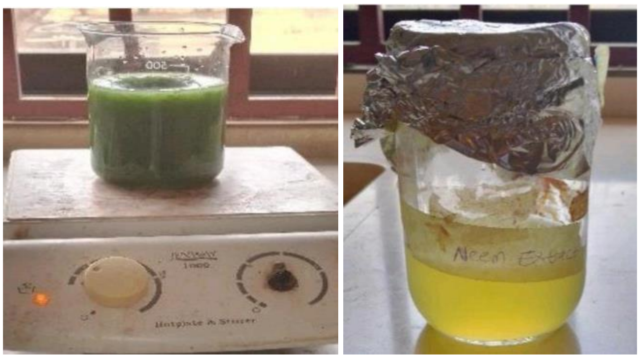

Figure 1. Synthesis of silver nanoparticles

Figure 2. Characteristic peak of silver nanoparticles absorption

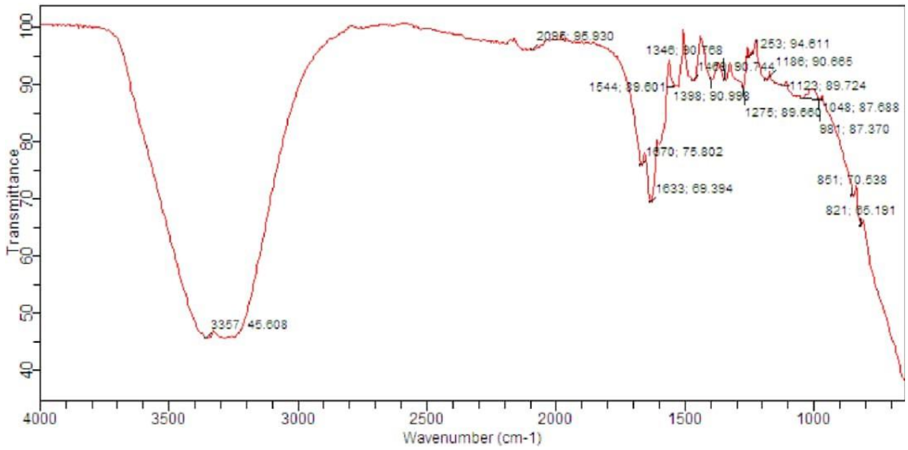

Figure 3. FTIR spectrum of synthesized AgNPs

Effects of experimental diets on serum biochemical parameters of albino rats at the end of 14 days feeding experiment

The result of the serum biochemical parameters (Figure 5) indicated that there were no significant differences (P>0.05) among the groups in the parameters estimated. Also, the effects of experimental diets on the performance of the experimental animals revealed that Group 1 had the highest feed efficiency ratio (0.26), while the lowest feed efficiency ratio of 0.14 was observed for Group 4. There was no significant difference (P>0.05) between Group 2 and Group 3 feed conversion ratio or feed efficiency, however, Groups 1 and 2 were significantly higher when compared with Group 4. However, there was no significant difference (P>0.05) in feed efficiency between Group 2 and 3.

Effects of experimental diet on performance of albino rats at the end of 14 days feeding experiment

The effects of the experimental diet on the growth Performance of the animals at the end of 14 days are shown in Table 1 The results revealed that AgNPs administration had no significant effect on average weekly weight gain, feed intake, and feed efficiency ratio. The average weekly feed intake and weight gain values of Groups 3 and 4 are higher than the Control Group. The result also revealed that the feed efficiency ratio decreased progressively with a significant difference from the Control Group to Group 4, while the feed conversion ratio increased across the group. There is no significant difference in the feed conversion ratio between Groups 2 and 3, however, these groups are significantly higher than the Control Group and at the same time significantly lower than Group 4 (Figure 4).

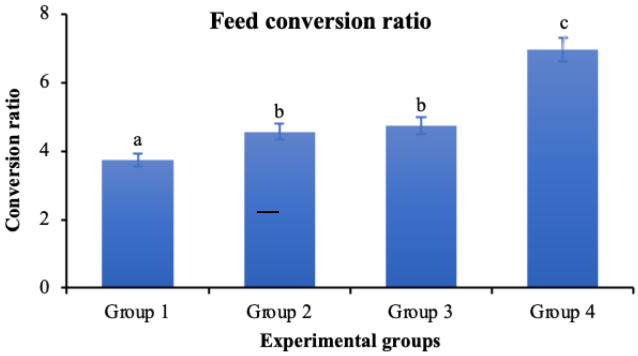

Figure 4. Feed conversion ratio of albino rats at the end of 14 days feeding experiment. Group valueswith different superscripts are significantly different (P<0.05) using descriptive analysis

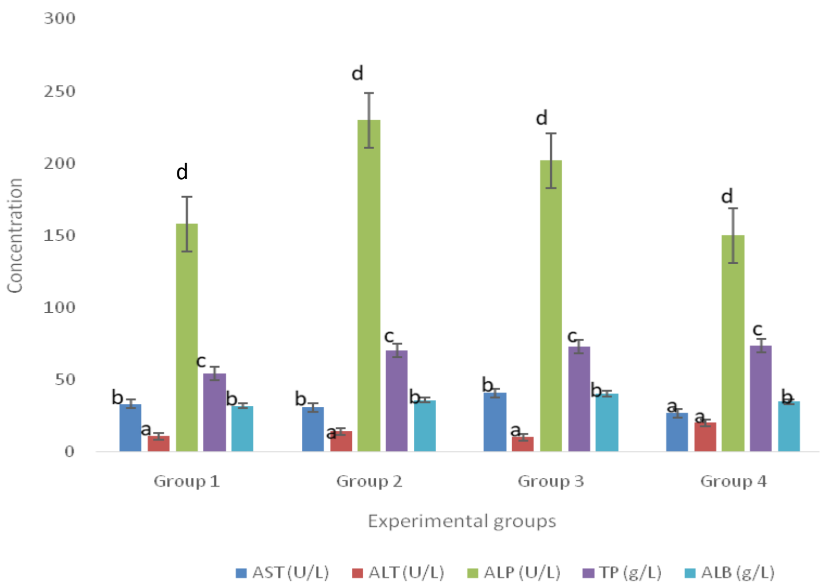

Figure 5. Effects of experimental diets on serum biochemical parameters of albino rats at the end of 14 days feeding experiment. Note: Values are presented as means ± SD for duplicate determinations, and values with different superscripts within the same column have no significant difference (P>0.05). Group 1 served as control and received potable water daily. Groups 2, 3, and 4 were daily administered with 100, 250, and 500 mg/kg AgNPs respectively for 12 days. Aspartate Aminotransferase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), Total Protein (TP), and Albumin (ALB)

Table 1. Effects of experimental diet on performance of albino rats at the end of 14 days feedingexperiment

Treatments	Group 1	Group 2	Group 3	Group 4
Average weekly Weight gain (g)	144.50±04	86.50±10	117.50±09	88.50±12
Average weekly Feed intake (g)	550.00±13	397.00±11	557.50±08	614.00±07
Feed efficiency Ratio	0.26±09^{a}	0.22±12^{b}	0.21±14^{b}	0.14±15^{c}

Discussion

An expanding field of study is the green production of nanoparticles, which is a well-known substitute for conventional techniques. This method utilizes plant extracts for the biosynthesis of nanoparticles. The advantages of green syntheses over chemical and physical approaches include environmental friendliness, cost-effectiveness, and ease of scaling up for large-scale nanoparticle production. In addition, there is no need to utilize harmful compounds or high temperatures, pressures, or energies (David et al. 2014). Taking into account the enormous potentiality of plants as a source, this work synthesized AgNPs using *A. indica* aqueous leaf extract.

In this study, silver nanoparticles were synthesized from plant material. The absorption characteristic peak of the ultraviolet-visible spectrum of the silver nanoparticles synthesized was observed at 445 nM (Figure 2). FTIR spectroscopy was used to identify the functional groups and biomolecules available in the crystal structure of silver nanoparticles (Figure 2). These biomolecues were identified based on the wavelength of their absorption and transmittance. The peak at this wavelength (3357 nM) was due to the absorption by hydroxyl group that could be found in flavonoids andpolyphenolics. This band appeared for AgNPS at transmittance of 45.608, the shift occurred is indicative of the chelative between silver nanoparticles via OH group, which could be alcohols i.e. aromatic alcohol. Unsaturated hydrocarbons could be present, since the stretches at 2095 nM could be due to the alkyne (c≡c) bond absorption, while 1670 and 1663 nM could be due to alkene (c═c) and alkanes within the crystal structure of Ag-NPs.

To study the effects of silver nanoparticles on the liver function enzymes and growth performance, the nanoparticles synthesized were administered to the experimental rats at different concentrations for 14 days. The result of the serum biochemical parameters (Figure 5) indicated that there were no significant differences (P>0.05) among the groups in the parameters estimated. Also, the effects of experimental diets on the performance of the experimental animals revealed that Group 1 had the highest feed efficiency ratio (0.26), while the lowest feed efficiency ratio of 0.14 was observed for Group 4. There was no significant difference (P>0.05) between Group 2 and Group 3 feed conversion ratio or feed efficiency, however, Groups 1 and 2 were significantly higher when compared with Group4. However, there was no significant difference (P>0.05) in feed efficiency between Groups 2 and 3.

The activity of AST and ALT enzymes is the first stage in examining liver damage, normally, an increase in AST and ALT indicates a liver problem (Gavanji et al. 2014). The most sensitive and the most practical recognizing enzymes in the liver are aminotransferases. The enzymes normally exist within the liver cells. When the liver gets damaged, the cells flow the enzymes to the blood and the increase in the level of the enzymes indicates liver damage (Reitman and Frankel 1957). ALP is a hydrolytic enzyme whose activity is observed in alkaline pH and different forms of these enzymes exist in the blood. The level of ALP serum in blood increases in pathologic conditions as well as in bone and liver damage (Soochan et al. 2012).

The average amounts of AST, ALT, and ALP were observed to be 33.50±3.54, 11.00±9.90, and 158.00.0±31.11 U/L respectively in the Control Group. The highest amount of AST (41.00±25.46 U/L), ALT (20.50±0.7), and ALP (230.00±0.00) were observed to be non-significant with a silver nanoparticle concentration of 250 mg/kg (Group 3), 500 mg/kg (Group 4), and 100 mg/kg (Group 2) respectively. These findings are similar to research carried out by Gavanji et al. (2014) who reported that there was no meaningful difference in AST and ALP levels in all groups, although, the level of ALT lead to a meaningful effect when compared with control. This result differs from that reported by Parang and Moghadamnia (2018) who observed a significant increase in the mean serum concentrations of AST, ALT, and ALP with an increase in silver nanoparticle concentrations. This could be attributed to the type of synthesis used and dosage of administered AgNPs. Also, the change in the mean of albumin and total protein levels in the experimental groups was observed to be non-significant. However, Parang and Moghadamnia (2018) reported a significant increase in all the treated groups compared to the Control Group.

Al Gurabi and associates in 2015 have looked into the possible impacts of silver nanoparticles on DNA damage and apoptotic cell death in albino mice in vivo (Ali et al. 2012). It has been demonstrated that silver nanoparticles significantly worsen liver injury symptoms, resulting in elevated ALP, ALT, and AST enzyme levels. Silver nanoparticles were employed to treat mice, and it was discovered that the particles caused DNA damage and cell death in lymphocytes and the liver. 7.8 mg/kg of silver nanoparticles significantly damaged DNA and resulted in cell death (Ali et al. 2012).

According to Guo et al. (2015), intravenous administration of silver nanoparticles causes the liver and kidneys to become toxic by weakening endothelial connections that are associated with intracellular ROS (Guo et al. 2015). Additionally, through intravenous exposure, alterations of endothelial cells brought on by silver nanoparticles can influence widespread peripheral inflammation in the liver and kidneys (Guo et al. 2015). Also, systemic exposure to silver nanoparticles was demonstrated to cause liver damage and NLRP3-dependent inflammation in a study by Ramadi et al. (2016). The concentrations of AST, ALT, and LDH were also raised by these nanoparticles. The findings of this study, which indicate that silver nanoparticles increase blood serum levels in a non-significant manner, are in agreement with some researchers' investigations, and also differ slightly from other studies.

The effects of the experimental diet on the growth Performance of the animals at the end of 14 days are shown in Table 1. The results revealed that AgNPs administration had no significant effect on average weekly weight gain, feed intake, and feed efficiency ratio. The average weekly feed intake and weight gain values of Groups 3 and 4 are higher than the Control Group. The result also revealed that the feed efficiency ratio decreased progressively with a significant difference from the Control Group to Group 4, while the feed conversion ratio increased across the group. There is no significant difference in the feed conversion ratio between Groups 2 and 3, however, these groups are significantly higher than the Control Group and at the same time significantly lower than Group 4. These significant differences could be caused by increasing levels of AgNPs suggesting their accumulation at different levels which may induce function alternation and may consequently affectthe metabolic rate of the body.

In conclusion, synthesized silver nanoparticles from *A. indica* altered the level of some selected biochemical parameters such as ALT, ALP, TP AST, and Albumin in a non-significant way. This study found no evidence of hazardous effects from silver nanoparticles, which could be attributed to the minimal dosage of AgNPs used or the source of the nanoparticles. Also, the growth performance of AgNPs administered rats showed a progressive significant increase in their feed conversion ratio, which may be attributed to varying concentration of AgNPs administered to the group.

REFERENCES

Ali D, Alkahtani S, Gurabi M, Alarifi S. 2012. In vivo DNA damaging and apoptotic potential of silver nanoparticles in Swiss albino mice. Onco Targets Ther 8: 295-302. DOI: 10.2147/OTT.S77572.

AOAC. 2000. Official Methods of Analysis 17th Edn. Association of Official Analysis Chemist (AOAC) International, Gaithersburg, M.D.

Chao JB, Liu JF, Yu SJ, Feng YD, Tan ZQ, Liu R, Yin YG. 2011. Speciation analysis of silver nanoparticles and silver ions in antibacterial products and environmental waters via cloud point extraction-based separation. Anal Chemother (83): 6875-6882. DOI: 10.1021/ac201086a

David L, Moldovan B, Vulcu A, Olenic L, Perde-Schrepler M, Fischer-Fodor E, Florea A, Crisan M, Chiorean I, Clichici S. 2014. Green synthesis, characterization and anti-inflammatory activity of silver nanoparticles using European black elderberry fruits extract. Colloids Surf B: Biointerfaces 122: 767-777. DOI: 10.1016/j.colsurfb.2014.08.018.

Dewi FRP, Lim V, Rosyidah A, Fatimah, Wahyuningsih SPA, Zubaidah U.2023. Characterization of silver nanoparticles (AgNPs) synthesized from *Piper ornatum* leaf extract and its activity against food borne pathogen *Staphylococcus aureus*. Biodiversitas 24: 1742-1748. DOI: 10.13057/biodiv/d240348.

Dziendzikowska K, Gromadzka-Ostrowska J, Lankoff A, Oczkowski M, Krawczynska A, Chwastowska J, Sadowska-Bratek M, Chajduk E, Wojewodzka M, Dusinska M, Kruszewski M. 2012. Time-dependent bio distribution and excretion of silver nanoparticles in male Wistar rats. JAppl Toxicol 32: 920-928. DOI: 10.1002/jat.2758.

Gavanji S, Sayedipour SS, Doostmohammadi M, Larki B. 2014. The effect of different concentrations of silver nanoparticles on enzyme activity and liver tissue of adult male wistar rats in-vivo condition. Intl J Sci Res Knowl 2 (4): 182-188. DOI: 10.12983/ijsrk-2014-p0182-0188.

Girish K, Shankara BS. 2008. Neem-a green treasure. Elec J Biol 4 (3): 102-111.

Gou Y, Zhou R, Ye X, Gao S, Li X. 2015. Highly efficient in vitro biosynthesis of silver nanoparticles using *Lysinibacillus sphaericus* MR-1 and their characterization. Sci Technol Adv Mater 16: 015004. DOI: 10.1088/1468-6996/16/1/015004.

Graf C, Vossen DL, Imhof A, Van-Blaaderen A. 2003. A general method to coat colloidal particles with silica. Langmuir 19 (17): 6693-6700. DOI: 10.1021/la0347859.

Guo H, Zhang J, Boudreau M, Meng J, Yin J-j, Liu J, Xu H. 2015. Intravenous administration of silver nanoparticles causes organ toxicity through intracellular ROS-related loss of inter-endothelial junction. Part Fibre Toxicol 13: 1. DOI: 10.1186/s12989-016-0133-9.

Gurunathan S, Kalishwaralal K, Vaidyanathan R, Venkataraman D, Pandian SRK, Muniyandi J, Hariharan N, Eom SH. 2009. Biosynthesis, purification and characterization of silver nanoparticles using *Escherichia coli*. Colloids Surf B: Biointerfaces 74 (1): 328-335. DOI: 10.1016/j.colsurfb.2009.07.048.

Hirn S, Semmler-Behnke M, Schleh C, Wenk A, Lipka J, Schäffler M, Takenaka S, Möller W, Schmid G, Simon U, Kreyling WG. 2011. Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration. Eur J Pharm Biopharm (77): 407-416. DOI: 10.1016/j.ejpb.2010.12.029.

Kahrilas GA, Wally LM, Fredrick SJ, Hiskey M, Prieto AL, Owens JE. 2014. Microwave-assisted green synthesis of silver nanoparticles using orange peel extract. ACS Sustain Chem Eng 2 (3): 367-376. DOI: 10.1021/sc4003664.

Kalishwaralal K, Banumathi E, Pandian SBRK, Deepak V, Muniyandi J, Eom SH. 2009. Silver nanoparticles inhibit VEGF induced cell proliferation and migration in bovine retinal endothelial cells. Colloids Surf B: Biointerfaces 73: 51-7. DOI: 10.1016/j.colsurfb.2009.04.025.

Kim S, Ryu DY. 2013. Silver nanoparticle-induced oxidative stress, genotoxicity and apoptosis in cultured cells and animal tissues. J Appl Toxicol 33: 78-89. DOI: 10.1002/jat.2792.

Kulthong K, Srisung S, Boonpavanitchakul K, Kangwansupamonkon W, Maniratanachote, R. 2010. Determination of silver nanoparticle release from antibacterial fabrics into artificial sweat. Part Fibre Toxicol Rev (7): 8. DOI: 10.1186/1743-8977-7-8.

Nowack B, Krug HF, Height M. 2011. 120 years of nanosilver history: Implications for policy makers. Environ Sci Technol J 45 (4): 1177-1183. DOI: 10.1021/es103316q.

Parang Z, Moghadamnia D. 2018. Effects of silver nanoparticles on the functional tests of liver and its histological changes in adult male rats. Nanomed Res J 3(3): 146-153. DOI: 10.22034/nmrj.2018.03.005.

Pulit J, Banach M, Kowalski Z. 2015. Nanosilver-making difficult decisions. Ecol Chem Engin 18 (2): 185-196. WOS: 000292468700003.

Ramadi KB, Mohamed YA, Al-Sbiei A, Almarzooqi S, Bashir G, Al Dhanhani A, Sarawathiamma D, Qadri S, Yasin J, Nemmar A, Fernandez-Cabezudo MJ, Haik Y, Al-Ramadi BK. 2016. Acute systemic exposure to silver-based nanoparticles induces hepatotoxicity and NLRP3-dependent inflammation. Nanotoxicology 10 (8): 1061-74. DOI: 10.3109/17435390.2016.1163743.

Reitman S, Frankel SA. 1957. A colorimetric method for determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. Am J Clin Pathol 28: 59-63. DOI: 10.1093/ajcp/28.1.56.

Sahayaraj K, Rajesh S. 2011. Bionanoparticles synthesis and anti-microbial applications. In: MéndezVilas A (eds). Science Against Microbial Pathogens: Communicating Current Research and Technological Advances Formatex Research Center, Spain.

Schleh C, Semmler-Behnke M, Lipka J, Wenk A, Hirn S, Schäffler M, Schmid G, Simon U, Kreyling WG. 2012. Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration. Nano-toxicol (6): 36-46. DOI: 10.3109/17435390.2011.552811.

Sheikpranbabu S, Kalishwaralal K, Venkataraman D, Eom SH, Park J, Gurunathan S. 2009. Silver nanoparticles inhibit VEGF-and IL-1b-induced vascular permeability via Src dependent pathway in porcine retinal endothelial cells. J Nanobiotechnol 7: 8. DOI: 10.1186/1477-3155-7-8.

Silver S, Phung LT, Silver G. 2006. Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. J Indust Microbiol Biotechnol (33): 627-634. DOI: 10.1007/s10295-006-0139-7.

Soochan D, Keough V, Wanless I, Molinari M. 2012. Intra and extra-hepatic cystadenoma of the biliary duct. Review of literature and radiological and pathological characteristics of a very rare case. BMJ Case Rep 20: 1-7. DOI: 10.1136/bcr.01.2012.5497.

Srivastava SK, Agrawal B, Kumar A, Pandey A. 2020. Phytochemicals of *Azadirachta indica* source of active medicinal constituent used for cure of various diseases: A review. J Sci Res 64 (1): 285-290. DOI: 10.37398/JSR.2020.640153.

Völker C, Oetken M, Oehlmann J. 2013. The biological effects and possible modes of action of nanosilver. Rev Environ Contam Toxicol 223: 81-106. DOI: 10.1007/978-1-4614-5577-6_4.

Wijnhoven SWP, Peijnenburg WJG, Herberts CA, Hagens WI, Oomen AG, Heugens EHW, Roszek B, Bisschops J, Gosens I, van de Meent D, Dekkers S, deJong WH, van Zijverden M, Sips AJAM, Geertsma RE. 2009. Nano-silver: A review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicol 3: 109-38. DOI: 10.1080/17435390902725914.

CELL BIOLOGY & DEVELOPMENT
Volume 7, Number 1, June 2023
Pages: 35-40

E-ISSN: 2580-4499
DOI: 10.13057/cellbioldev/v070105

Protective effect of vitamin C against alcohol induced lungs toxicity in adult male Wistar rats

OLUSOJI A. OYESOLA, IFABUNMI O. OSONUGA, EMMANUEL T. GEORGE♥
Department of Physiology, Faculty of Basic Medical Sciences, Olabisi Onabanjo University. Ogun State, Nigeria. Tel.: +234-8055606475,
♥email: georgeayoku@gmail.com.

Manuscript received: 13 April 2023. Revision accepted: 30 June 2023.

Abstract. *Oyesola OA, Osonuga IO, George ET. 2023. Protective effect of vitamin C against alcohol induced lungs toxicity in adult male Wistar rats. Cell Biol Dev 7: 35-40.* The lungs are the major organs in the respiratory system. The toxic effect of alcohol on the lungs leads to cell death and loss of function. This study aimed to investigate the protective effects of vitamin C on alcohol-induced lung toxicity in male Wistar rats. Forty male Wistar rats were acclimated for 14 days and randomly divided into eight groups. Group A was the control and received only distilled water. Group B was given alcohol, while groups C, D, and E received varying doses of vitamin C. Groups F, G, and H received alcohol, followed by vitamin C. After 21 days of treatment, the rats' lungs were collected and evaluated for various parameters, including antioxidant enzyme activity (Catalase (CAT), Glutathione (GSH), and Superoxide Dismutase (SOD), lipid peroxidation levels (malodialdehyde (MDA), carbon dioxide (CO_2) levels in the blood, and histopathological changes. The results indicated that rats that received only alcohol had increased lipid peroxidation (MDA) levels, reduced antioxidant enzyme activity (CAT, SOD, and GSH) in the lungs, high CO_2 levels in the blood, dilation of the alveolar sac, and disorientation of the bronchioles. However, groups treated with alcohol and vitamin C exhibited increased antioxidant enzyme activity, reduced lipid peroxidation and CO_2 content of the blood, regenerative changes, and improvement in the histo-architecture of the lungs. Vitamin C has demonstrated protective properties against alcohol-induced lung toxicity.

Keywords: Antioxidants, carbon dioxide, lung, vitamins

INTRODUCTION

Alcohol consumption is a widespread global practise, and the effect of its consumption depends on the quantity and quality of alcohol consumed. Studies have suggested that moderate consumption of alcohol may reduce the risk of developing cardiovascular diseases (Rehm et al. 2017); other research has also found no clear relationship between the level of alcohol consumption and the cardiovascular system (Weng and Dunn 2019). However, the consumption of alcohol is related to the development of various diseases such as cancer, liver diseases, cardiovascular diseases, diabetes, and neuropsychiatric diseases (Rungratanawanich et al. 2021). The development of these above-mentioned diseases may be determined by genetic predisposition, malnutrition, and concurrent viral infection of the liver (Neuman et al. 2014; Teschke 2019).

Vitamins are important for various physiological and biochemical processes in the human body (Wishart 2019). Human beings need to get vitamins from external sources, such as diet, since they can't synthesise them (Capozzi et al. 2012). Vitamin C is an important water-soluble compound that is essential for many biological processes, including tissue repair, collagen formation, immune system function, increasing antioxidant enzyme activity, and the production and functioning of several enzymes (Lykkesfeldt and Tveden-Nyborg 2019). Vitamin C also plays an important role in enzymatic reactions, the manufacture of hormones and neurotransmitters, and other biological processes.

The lungs are important organs of the respiratory system that allow gas exchange between the human body and the external environment (Molnar and Gair 2013). Excessive consumption of alcohol can lead to the development of respiratory diseases such as Acute Respiratory Distress Syndrome (ARDS), Chronic Obstructive Pulmonary Disease (COPD), and pneumonia (Szabo and Saha 2015). Although the liver is responsible for the metabolism of ingested alcohol, a small percentage of the ingested alcohol travels through the bronchial circulation to the airway passage, where it undergoes oxidative and non-oxidative processes. Some alcohol may be expelled unaltered through breathing. The consumption of alcohol leads to weakness of the systemic immune system, making alcohol drinkers more vulnerable to lung infections with severe symptoms and unfavorable consequences such as ARDS and COPD (Kaphalia and Calhoun 2013). The consumption of alcohol may also lead to a deficiency of vitamin C. As mentioned above, vitamin C has antioxidant activity, which counters the oxidative stress induced by alcohol. It also has the ability to improve the production of collagen, a structural protein that supports the lungs and is vital in maintaining the elasticity of the lungs to function normally. Previous studies have shown that vitamin C has the ability to reduce the risk of respiratory infection by enhancing the immune activity of T cells and phagocytes, which fight off infections.

Additionally, vitamin C has been found to improve lung function and reduce airway inflammation in patients with asthma, a chronic respiratory disease characterized by airway inflammation and constriction (Schloss et al. 2020). This present study will evaluate the antioxidant and anti-inflammatory activity of vitamin C against alcohol toxicity in adult male Wistar rats.

MATERIALS AND METHODS

Animal care and grouping

For this experiment, forty (40) adult healthy Wistar male rats weighing 150g to 250g were utilized. The rats were housed in wire and plastic cages in the Olabisi Onabanjo University animal house at the Obafemi Awolowo College of Health Sciences, Sagamu Campus, Ogun State, Nigeria. The rats were given two weeks to acclimatize; they were fed a standard pellet diet and given unrestricted access to water. The National Research Council's (2011) internationally recognized standard rules for the use of animals were followed in the handling and care of the animals.

Ethical approval for the use and care of laboratory animals was obtained from the Ethical Committee for Research of the Department of Physiology, Faculty of Basic Medical Science (FBMS), Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria, with approval number OOU/PHSECR/22/009.

Eight groups of five rats each were formed randomly from the rat population, and each group received treatments for 21 days. All treatement was administered through the oral route of administration using an oral gavage.

Group A: Distilled water only

Group B: 6000 mg/kg body weight of alcohol (30% v/v)

Group C: 100 mg/kg body weight of vitamin C

Group D: 200 mg/kg body weight of vitamin C

Group E: 300 mg/kg body weight of vitamin C

Group F: 6000 mg/kg body weight of alcohol (30%v/v) and 100 mg/kg body weight of vitamin C

Group G: 6000 mg/kg body weight of alcohol (30% v/v) and 200 mg/kg body weight of vitamin C

Group H: 6000 mg/kg body weight of alcohol (30% v/v) and 200 mg/kg body weight of vitamin C

Procedure for blood collection and determination of blood CO_2 levels

Blood was collected from the retro-orbital sinus, six hours after the admiration of the last treatment, after blood collection, the sample was centrifuged at 1200rpm for fifteen minutes, the supernatant was then analysed for CO_2 level using an automated electrolyte analyser (SFRI ISE6000-France)

Procedure for determination of antioxidant enzymes activity and lipid peroxidation level of the lungs

The lungs tissue to be accessed for oxidative stress and level of lipid peroxidation, was homogenized in phosphate buffer. Glutathione Reductace (GSH) activity of the lungs was determined using the method described by Sedlak and Lindsay (1968). Catalase (CAT) activities of the lungs was determined by the method described by Sinha (1972), while Superoxide Dismutase (SOD) activity of the lungs was determined accoding to the method of Sun and Zigman (1978). Level of lipid peroxidation (malondiadehyde, MDA) was measure accoding to the methods of Buege and Aust (1978).

Histological examination

After harvesting the lung tissues, it was fixed in a 10% neutral buffered formalin, it was later embedded in paraffin and 5 µm thick sections were prepared and stained with heamatoxylin and eosin using standard procedures. The slides were viewed under light microscope (CELESTRON LCD DIGITAL MICROSCOPE, MODEL 44348) and photomicrographs were taken (200×)

Statistical analysis

All analysis was done using SPSS (version 16) and Microsoft Excel (2019) using and student T-test. Data were expressed as Mean± SEM with $p<0.05$ considered statistically significant. in the results section;

[a]-Values were significant when compared to Group A,
[b]-Values were significant when compared to Group B,
[c]-Values were significant when compared to Group C,
[d]-Values were significant when compared to Group D,
[e]-Values were significant when compared to Group E,
[f]-Values were significant when compared to Group F,
[g]-Values were significant when compared to Group G.

RESULTS AND DISCUSSION

Protective effect of vitamin C against alcohol induced pathological changes on the CO_2 level in adult male Wistar rats

Figure 1 represents the protective activity of vitamin C against alcohol-induced pathological changes in the CO_2 concentration in male Wistar rats. The CO_2 levels in test groups D and E were significantly lower than those in the control group, while the levels in test Group B were significantly higher when compared to other groups, including the control group. When compared to test groups C, D, E, F, G, and H, there was a significant decrease in the carbon dioxide level of rats administered with 6000 mg/kg of alcohol. In rats administered 100 mg/kg of vitamin C, there was a significant decrease in the plasma carbon dioxide level when compared to test groups D, E, and H. There was a significant increase in the plasma carbon dioxide level of rats administered with 300 mg/kg of vitamin C when compared to test Group F.

Metabolic acidosis is a condition that arises when the body generates too much acid or when the organs that are responsible for the elimination of acidic radicals cannot remove or neutralise enough acid from the body. Acute or chronic intoxication from acid-producing substances, including alcohol, chemicals, and some acidiosis-forming medications, is mainly responsible for metabolic acidiosis. The human body has multifactorial physiological mechanisms to neutralise these acids and remove them from the body via Carbon Dioxide (CO_2) and the Bicarbonate Ion (HCO_3^-), thereby regulating the pH of the blood (Melamed and Melamed 2014). When the CO_2 levels in the blood are high, the concentration of the H^+ ions in the body increases, which will lead to the lowering of the blood pH, causing the pathological state of metabolic acidosis. In our study, the groups administered with alcohol only showed an increase in CO_2 in the blood; this indicated alcohol-induced hypercapnia, which corresponds with the previous study of Sabino et al. (2014). Vitamin C, on the other hand, has a protective effect on the lungs by decreasing systemic oxidative stress, increasing nitric oxide bioavailability, and also restoring vascular endothelial function (Hartmann et al. 2015; Carr and Maggini 2020). The protective effect of vitamin C can be seen across the coadministration Group And in the vitamin C group only.

Protective effect of vitamin C against alcohol induced oxidative stress in the lungs tissue of male Wistar rats

The graphs in Figures 2-5 show the antioxidant effect of vitamin C against alcohol-induced oxidative stress and an increase in lipid peroxidation in the lung tissues of male Wistar rats. The results showed that the consumption of alcohol led to a significant decrease in the antioxidant enzyme activity of the lungs (Figures 2-4) and an increase in the level of lipid peroxidation (Figure 5). In contrast, groups treated with vitamin C showed a significant increase in antioxidant enzyme activity and a decrease in lipid peroxidation levels. Group H, which received the highest dose of vitamin C after alcohol treatment, showed the most significant improvement in antioxidant enzyme activity and levels of lipid peroxidation in comparison to other groups. Oxidants are generated endogenously by metabolic reactions or derived from exogenous sources, and biological systems are constantly exposed to them (Bhattacharya 2015). The human lungs are exposed to a high level of oxygen, which makes them highly susceptible to oxidative injury mediated by free radicals, together with their large surface area and blood supply (Lodovici and Bigagli 2011). The high level of oxidant and the low level of antioxidant will lead to the oxidation of DNA molecules, proteins, and lipids in the cell, as well as inducing different cellular responses through the generation of secondary metabolic free radicals (Di Rosanna and Salvatore 2012). Recent studies have shown that oxidative stress plays an important role in the generation and development of various respiratory pathologies, including asthma, COPD, acute lung injury, lung cancer, and pulmonary fibrosis (Di Rosanna and Salvatore 2012; Liu and Chen 2017). The consumption of alcohol increases oxidative stress through different mechanisms, including the generation of superoxide anion and the production of free radicals at the microsomal level (Jing et al. 2012). According to Table 1 below, the administration of alcohol leads to a decrease in the level of CAT, SOD, and GSH enzyme activity in the lungs. This reduction is also in line with the study of Macdonald et al. (2010). The consumption of alcohol not only activates free radicals but also alters the level of enzymatic and non-enzymatic endogenous antioxidant systems; this will result in oxidative stress with a cascade of effects leading to pathological changes in both the functional and structural integrity of the cell and its organelle membrane (DeLeve et al. 1996), which can also be seen in plate 1b below. The disruption seen in plate 1b below can also be linked to the high level of lipid peroxidation, which will affect the lipid layer of the cell membrane and can only be seen in the alcohol groups. This shows that the consumption of alcohol leads to the disruption of the antioxidant defense system.

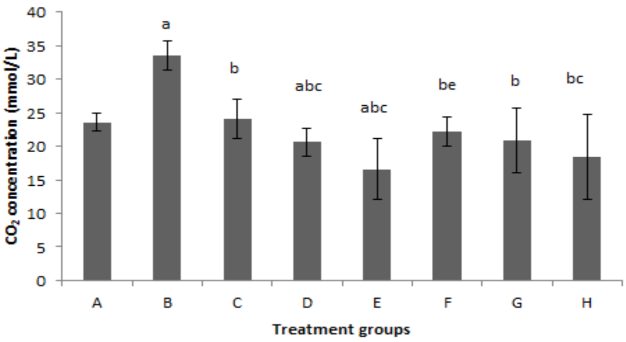

Figure 1. Protective effect of vitamin C against alcohol induced pathological changes on the CO_2 level in adult male Wistar rats. Note: Each bar is an expression of mean ± SEM. (P <0.05). A. Values were significant when compared to Group A, B. Values were significant when compared to Group B, C. Values were significant when compared to Group C, D. Values were significant when compared to Group D, E. Values were significant when compared to Group E, F. Values were significant when compared to Group F, G. Values were significant when compared to Group G

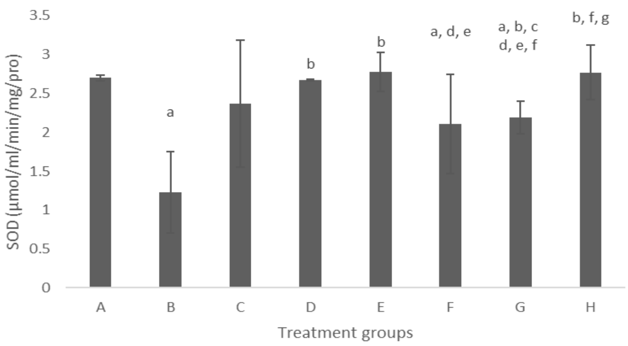

Figure 2. Protective effect of vitamin C against alcohol induced pathological changes on the SOD activity in the lungs tissue of male Wistar rats. Note: Each bar is an expression of mean ± SEM. (P <0.05). A. Values were significant when compared to Group A, B. Values were significant when compared to Group B, C. Values were significant when compared to Group C, D. Values were significant when compared to Group D, E. Values were significant when compared to Group E, F. Values were significant when compared to Group F, G. Values were significant when compared to Group G

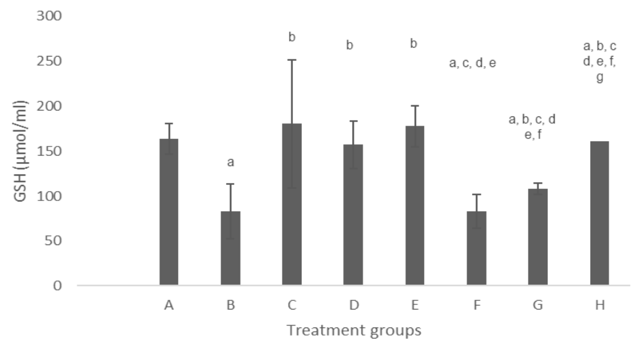

Figure 3. Protective effect of vitamin C against alcohol induced pathological changes on the GSH activity in the lungs tissue of male Wistar rats. Note: Each bar is an expression of mean ± SEM. (P <0.05). A. Values were significant when compared to Group A, B. Values were significant when compared to Group B, C. Values were significant when compared to Group C, D. Values were significant when compared to Group D, E. Values were significant when compared to Group E, F. Values were significant when compared to Group F, G. Values were significant when compared to Group G

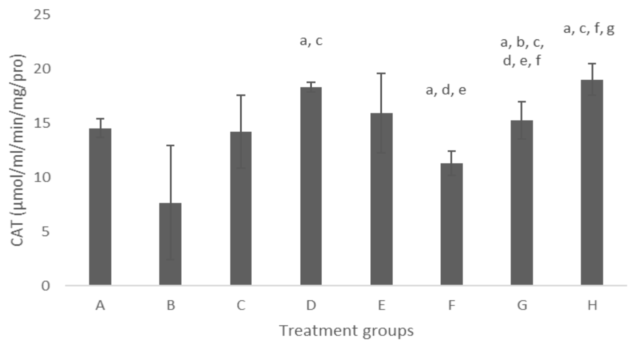

Figure 4. Protective effect of vitamin C against alcohol induced pathological changes on the CAT activity in the lungs tissue of male Wistar rats. Note: Each bar is an expression of mean ± SEM. (P <0.05). A. Values were significant when compared to Group A, B. Values were significant when compared to Group B, C. Values were significant when compared to Group C, D. Values were significant when compared to Group D, E. Values were significant when compared to Group E, F. Values were significant when compared to Group F, G. Values were significant when compared to Group G

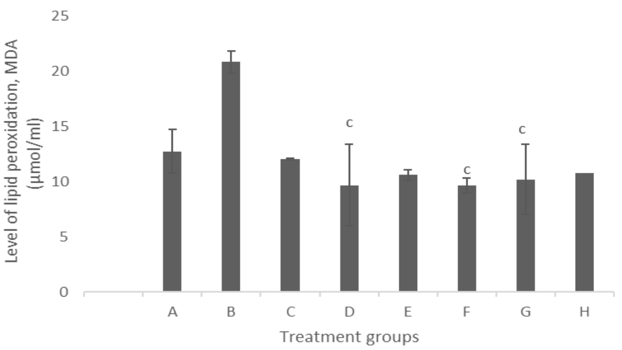

Figure 5. Protective effect of vitamin C against alcohol induced pathological changes on the level of lipid peroxidation (MDA) in the lungs tissue of male Wistar rats. Note: Each bar is an expression of mean ± SEM. (P <0.05). A. Values were significant when compared to Group A, B. Values were significant when compared to Group B, C. Values were significant when compared to Group C, D. Values were significant when compared to Group D, E. Values were significant when compared to Group E, F. Values were significant when compared to Group F, G. Values were significant when compared to Group G

A therapeutic approach to alcohol-induced oxidative stress may involve the use of exogenous and endogenous antioxidant entities (Xu et al. 2020). Recent research involving the use of antioxidants such as vitamin C and E has shown that these vitamins to an extent exert antioxidative and anti-inflammatory activity on lung tissue (Domej et al. 2014; Adewoyin et al. 2017; Liu et al. 2018). The results of our study showed that the administration of vitamin C has both antioxidant and inflammatory effects on alcohol-induced lung toxicity.

Protective effect of vitamin C against alcohol induced tissue damage on the histo- architecture of the lungs in male Wistar rats

The toxic effect of alcohol on the respiratory system is underappreciated. This is due to the fact that there are no pathological changes noticed at first until there is a secondary insult. Chronic ethanol ingestions impact all aspects of the alveolar epithelium. This is due to the fact that the alveoli are rich in blood supply, alcohol is absorbed and distributed in an unaltered state, meaning it is not bound to any protein or transported via any specific transport mechanisms, and the lungs are the most vulnerable organs after the ingestion of alcohol (Downs et al. 2013). The study of Brown and Brown (2012) revealed that chronic consumption of alcohol will lead to a pathological impairment in alveolar macrophage function that will further degenerate into decreased phagocytosis and increased reactive oxygen species production, as seen in Table 1.

In Figure 6.B, there was a dilated alveolar sac, which indicated that the walls of the airspace below the terminal bronchioles were damaged. When the alveoli are dilated, the structure of the alveoli is destroyed, the elasticity is reduced and lost, leading to air stagnation, which will lead to the impairment of the gas exchange function of the alveoli. The dilation of the alveoli will also complement the alcohol-induced bronchioles disorientation. These pathological changes are caused by an Alpha-1 Antitrypsin (AAT) protein deficiency. Previous studies have shown that the consumption of alcohol is responsible for the deficiency of AAT proteins; the AAT proteins have a protective effect on the elasticity of the alveoli, and their deficiency will lead to dilation of the alveoli (Senn et al. 2008; Hoth et al. 2012). Since alcohol also plays an important role in the development of oxidative stress (Kahraman et al. 2012), according to the study of Dasi et al. (2013), oxidative stress also leads to a deficiency of the AAT protein.

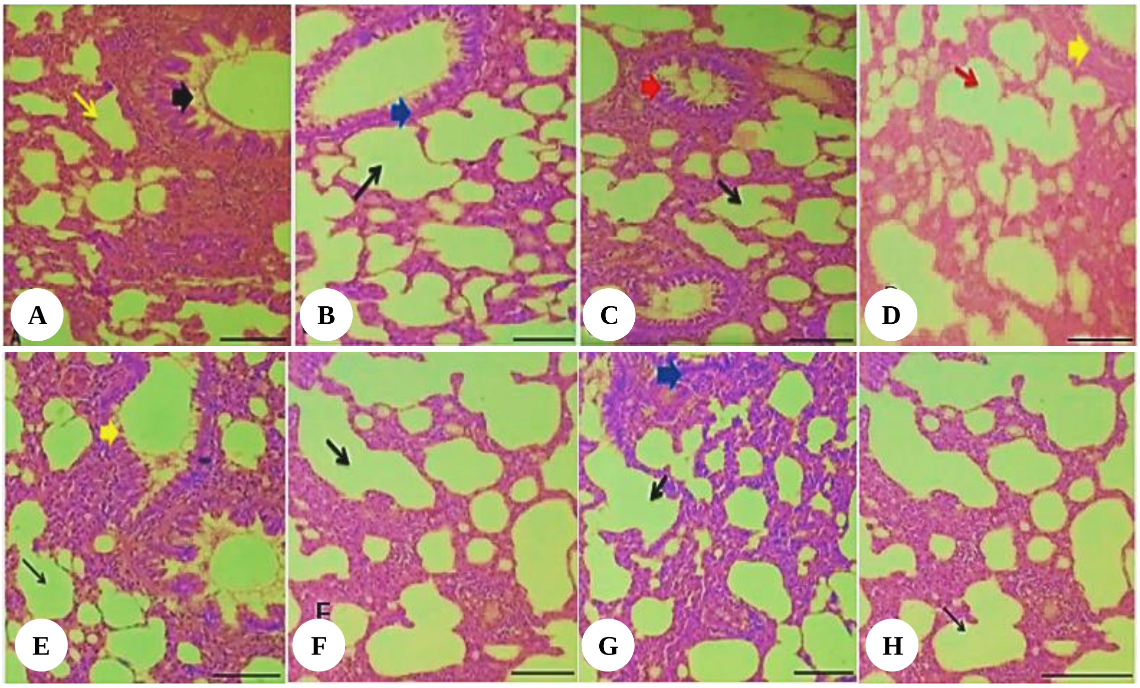

Figure 6. Protective effect of vitamin C against alcohol induced tissue damage on the histo- architecture of the lungs in male Wistar rats H/E X200. Scale Bar =120µm

The toxic effect of free radicals can be canceled out by antioxidants, which may be dietary, endogenous, enzymatic, or non-enzymatic, through various mechanisms, such as electron donation, catalytic removal, binding radicals, and gene expression regulation. Together, antioxidants constitute an integrated defense against ROS and the development of oxidative stress (Janciauskiene 2020). Previous studies have shown that vitamin C and other classes of antioxidants have a protective effect on lung function (Grievink et al. 1998; Ares et al. 2013). In our study, the administration of vitamin C caused regenerative changes in the histo-architecture of the lungs. This is due to the fact that vitamin C works by increasing Nrf2 expression and protein levels and, concomitantly, increasing the activity of antioxidant defense.

Figure 6 shows the protective effect of vitamin C against alcohol-induced lung toxicity. In the control group's lung tissue, there was a well-differentiated bronchiole (black thick arrow), alveolar sac (yellow thin arrow), and alveolar septa. In the group treated with 6000 mg/kg of alcohol, there was a dilated alveolar sac (black thin arrow) and disorientation of the bronchiole (blue thick arrow) with loss of function. In test Group C, there were no pathological histomorphological changes; the bronchiole (red thick arrow) and the alveoli sac (black thin arrow) are well organized. Test Group D has no pathological histomorphological changes; the bronchiole (yellow thick arrow) and the alveoli sac (red thin arrow) are well organized. Also, test Group E showed no pathological histomorphological changes; the bronchiole (yellow thick arrow) and the alveoli sac (black thin arrow) are well organized. In Group F, there was well-regenerated and improved lung tissue with a slight dilation of the alveoli sack. Test Group G showed well-regenerated and improved lung tissue with slight dilation of the alveoli sac (black thin arrow), while test group H showed well-regenerated and improved lung tissue with slight dilation of the alveoli sac (black thin arrow).

In conclusion, the consumption of alcohol has been shown to cause an increase in oxidative stress, which will lead to a decrease in antioxidant enzyme activity. In our study, vitamin C showed a protective effect against alcohol-induced toxicity in the lungs of adult male Wistar rats.

REFERENCES

Adewoyin M, Ibrahim M, Roszaman R, Md Isa, ML, Mat Alewi NA, Abdul Rafa AA, Anuar MNN. 2017. Male infertility: The effect of natural antioxidants and phytocompounds on seminal oxidative stress. Diseases 5 (1): 9. DOI: 10.3390/diseases5010009.

Ares AM, Nozal MJ, Bernal J. 2013. Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds. J Chromatogr A 1313: 78-95. DOI: 10.1016/j.chroma.2013.07.051.

Bhattacharya S. 2015. Reactive oxygen species and cellular defense system. In: Rani V, Yadav U (eds). Free Radicals in Human Health and Disease. Springer, New Delhi. DOI: 10.1007/978-81-322-2035-0_2.

Brown SD, Brown LAS. 2012. Ethanol (E t OH)-induced TGF-β1 and reactive oxygen species production are necessary for E t OH-induced alveolar macrophage dysfunction and induction of alternative activation. Alcohol Clin Exp Res 36 (11): 1952-1962. DOI: 10.1111/j.1530-0277.2012.01825.x.

Buege JA, Aust SD. 1978. [30] Microsomal lipid peroxidation. Methods Enzymol 52: 302-310. DOI: 10.1016/s0076-6879(78)52032-6.

Capozzi V, Russo P, Dueñas MT, López P, Spano G. 2012. Lactic acid bacteria producing B-group vitamins: A great potential for functional cereals products. Appl Microbiol and Biotechnol 96: 1383-1394. DOI: 10.1007/s00253-012-4440-2.

Carr AC, Maggini S. 2017. Vitamin C and immune function. Nutrients 9 (11): 1211. DOI: 10.3390/nu9111211.

Dasí F, Amor M, Sanz F, Codoñer-Franch P, Navarro-García MM, Escribano A. 2013. Oxidative stress in serum of patients with alpha-1 antitrypsin deficiency. Eur Respir J 42: 57.

DeLeve LD, Wang X, Kuhlenkamp JF, Kaplowitz N. 1996. Toxicity of azathioprine and monocrotaline in murine sinusoidal endothelial cells and hepatocytes: The role of glutathione and relevance to hepatic venoocclusive disease. Hepatology 23 (3): 589-599. DOI: 10.1002/hep.510230326.

Di Rosanna P, Salvatore C. 2012. Reactive oxygen species, inflammation, and lung diseases. Curr Pharm Design 18 (26): 3889-3900. DOI: 10.2174/138161212802083716.

Domej W, Oettl K, Renner W. 2014. Oxidative stress and free radicals in COPD–implications and relevance for treatment. Intl J Chron Obstr Pulm Dis 9: 1207-1224. DOI: 10.2147/COPD.S51226.

Downs CA, Trac D, Brewer EM, Brown LA, Helms MN. 2013. Chronic alcohol ingestion changes the landscape of the alveolar epithelium. BioMed Res Intl 2013: 470217. DOI: 10.1155/2013/470217.

Grievink L, Smit HA, Ocké MC, van‘t Veer P, Kromhout D. 1998. Dietary intake of antioxidant (pro)-vitamins, respiratory symptoms and pulmonary function: The MORGEN study. Thorax 53 (3): 166-171. DOI: 10.1136/thx.53.3.166.

Hartmann SE, Kissel CK, Szabo L, Walker BL, Leigh R, Anderson TJ, Poulin MJ. 2015. Increased ventilatory response to carbon dioxide in COPD patients following vitamin C administration. ERJ Open Res 1 (1): 00017-2015. DOI: 10.1183/23120541.00017-2015.

Hoth KF, Ford DW, Sandhaus RA, Strange C, Wamboldt FS, Holm KE. 2012. Alcohol use predicts ER visits in individuals with Alpha-1 Antitrypsin Deficiency (AATD) associated COPD. Intl J Chron Obstr Pulm Dis 9 (4): 417-425. DOI: 10.3390/jcm11133594.

Janciauskiene S. 2020. The beneficial effects of antioxidants in health and diseases. Chronic Obstr Pulm Dis 7 (3): 182. DOI: 10.15326/jcopdf.7.3.2019.0152.

Jing L, Jin CM, Li SS, Zhang FM, Yuan L, Li WM, Sang Y, Li S, Zhou LJ. 2012. Chronic alcohol intake-induced oxidative stress and apoptosis: role of CYP2E1 and calpain-1 in alcoholic cardiomyopathy. Mol Cell Biochem 359: 283-292. DOI: 10.1007/s11010-011-1022-z.

Kahraman A, Çakar H, Köken T. 2012. The protective effect of quercetin on long-term alcohol consumption-induced oxidative stress. Molecular Biol Rep 39: 2789-2794. DOI: 10.1007/s11033-011-1037-2.

Kaphalia L, Calhoun WJ. 2013. Alcoholic lung injury: Metabolic, biochemical and immunological aspects. Toxicol Lett 222 (2): 171-179. DOI: 10.1016/j.toxlet.2013.07.016.

Liu X, Chen Z. 2017. The pathophysiological role of mitochondrial oxidative stress in lung diseases. J Transl Med 15 (1): 1-13. DOI: 10.1186/s12967-017-1306-5.

Liu Z, Ren Z, Zhang J, Chuang CC, Kandaswamy E, Zhou T, Zuo L. 2018. Role of ROS and nutritional antioxidants in human diseases. Front Physiol 9: 477. DOI: 10.3389/fphys.2018.00477.

Lodovici M, Bigagli E. 2011. Oxidative stress and air pollution exposure. J Toxicol 2011: 487074. DOI: 10.1155/2011/487074.

Lykkesfeldt J, Tveden-Nyborg P. 2019. The pharmacokinetics of vitamin C. Nutrients 11 (10): 2412. DOI: 10.3390/nu11102412.

Macdonald IO, Olusola OJ, Osaigbovo UA. 2010. Effects of chronic ethanol administration on body weight, reduced Glutathione (GSH), Malondialdehyde (MDA) levels and glutathione-s-transferase activity (GST) in rats. NY Sci J 3 (4): 3947.

Melamed P, Melamed F. 2014. Chronic metabolic acidosis destroys pancreas. J Pancreas 15 (6): 552-560. DOI: 10.6092/1590-8577/2854.

Molnar C, Gair J. 2013. 11.3 Circulatory and Respiratory Systems. Concepts of Biol-1st Canadian Edition. BCcampus. Retrieved from https://opentextbc.ca/biology/

National Research Council. 2011. Guide for the Care and Use of Laboratory Animals. The National Academies Press, Washington D.C.

Neuman MG, French SW, French BA, Seitz HK, Cohen LB, Mueller S, Osna NA, Kharbanda KK, Seth D, Bautista A, Thompson KJ. 2014. Alcoholic and non-alcoholic steatohepatitis. Exp Mol Pathol 97 (3): 492-510. DOI: 10.1016/j.yexmp.2014.09.005.

Rehm J, Gmel Sr GE, Gmel G, Hasan OS, Imtiaz S, Popova S, Probst C, Roerecke M, Room R, Samokhvalov AV, Shield KD. 2017. The relationship between different dimensions of alcohol use and the burden of disease—an update. Addiction 112 (6): 968-1001. DOI: 10.1111/add.13757.

Rungratanawanich W, Qu Y, Wang X, Essa MM, Song BJ. 2021. Advanced Glycation End Products (AGEs) and other adducts in aging-related diseases and alcohol-mediated tissue injury. Exp Mol Med 53 (2): 168-188. DOI: 10.1038/s12276-021-00561-7.

Sabino JPJ, Silva ALD, Resstel LB, Antunes-Rodrigues J, Glass ML, Branco LG. 2014. Effect of chronic ethanol exposure on rat ventilatory responses to hypoxia and hypercapnia. Clinics 69: 360-366. DOI: 10.6061/clinics/2014(05)11.

Schloss J, Lauche R, Harnett J, Hannan N, Brown D, Greenfield T, Steel A. 2020. Efficacy and safety of vitamin C in the management of acute respiratory infection and disease: A rapid review. Adv Integr Med 7(4): 187-191. DOI: 10.1016/j.aimed.2020.07.008.

Sedlak J, Lindsay RH. 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25: 192-205. DOI: 10.1016/0003-2697(68)90092-4.

Senn O, Russi EW, Schindler C, Imboden M, von Eckardstein A, Brändli O, Zemp E, Ackermann-Liebrich U, Berger W, Rochat T, Luisetti M. 2008. Circulating alpha1-antitrypsin in the general population: Determinants and association with lung function. Respir Res 9 (1): 1-10. DOI: 10.1186/1465-9921-9-35.

Sinha AK. 1972. Colorimetric assay of catalase. Anal Biochem 47 (2): 389-394. DOI: 10.1016/0003-2697(72)90132-7.

Sun M, Zigman S. 1978. An improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. Anal Biochem 90 (1) 81-89. DOI: 10.1016/0003-2697(78)90010-6.

Szabo G, Saha B. 2015. Alcohol’s effect on host defense. Alcohol Res Curr Rev 37 (2): 159.

Teschke R. 2019. Alcoholic liver disease: Current mechanistic aspects with focus on their clinical relevance. Biomed 7 (3): 68. DOI: 10.3390/biomedicines7030068.

Weng G, Dunn W. 2019. Effect of alcohol consumption on nonalcoholic fatty liver disease. Transl Gastroenterol Hepatol 4: 70. DOI: 10.21037/tgh.2019.09.02.

Wishart DS. 2019. Metabolomics for investigating physiological and pathophysiological processes. Physiol Rev 99 (4): 1819-1875. DOI: 10.1152/physrev.00035.2018.

Xu Y, Liu H, Song L. 2020. Novel drug delivery systems targeting oxidative stress in chronic obstructive pulmonary disease: A review. J Nanobiotechnol 18 (1): 1-25. DOI: 10.1186/s12951-020-00703-5.

CELL BIOLOGY & DEVELOPMENT
Volume 7, Number 1, June 2023
Pages: 41-46
E-ISSN: 2580-4499
DOI: 10.13057/cellbioldev/v070106

Inhibitory efficacy of selected botanical, microbial and synthetic pesticides against *Colletotrichum alatae*, causing water yam anthracnose disease

ELIAS MJAIKA NDIFON
Department of Crop Science, Faculty of Agriculture, Alex Ekwueme Federal University Ndufu-Alike. PMB 1010 Abakaliki, Ebonyi State, Nigeria.
Tel.: +234-8085736651, email: emndi4nn@yahoo.com

Manuscript received: 8 May 2023. Revision accepted: 30 June 2023.

Abstract. *Ndifon EM. 2023. Inhibitory efficacy of selected botanical, microbial and synthetic pesticides against* Colletotrichum alatae, *causing water yam anthracnose disease. Cell Biol Dev 7: 41-46.* Yam (*Dioscorea* spp.) ranks first followed by cassava and sweet potato among root and tuber crops in sub-Sahara Africa. However, yam production is constrained mainly by diseases caused by fungi, bacteria, and nematodes. The aim of this research was to propose some practicable botanical, chemical, and biocontrol measures appropriate to the management of *Colletotrichum alatae,* causing tuber rot disease in water yam. All the experiments were conducted using a completely randomized design. The results revealed that Carbendazim+Mancozeb (50% concentration), Mancozeb+Cu(I)O+Metalaxyl (100% concentration), and Mancozeb (100% concentration) significantly ($P \leq 0.05$) inhibited the pathogen compared to the other treatments. *Trichoderma harzianum* isolate AIM16 showed significantly ($P \leq 0.05$) better inhibition of *C. alatae*. *T. harzianum* isolate NSBM, *T. harzianum* isolate BGMZ4, and *T. harzianum* isolate AIM3 were significantly ($P \leq 0.05$) better that the control. Extract of *Eucalyptus globulus* L. (100% concentration) showed the best inhibitory ability and was significantly different ($P \leq 0.05$) from other treatments, followed by *E. globulus* (50%), *Guieria senegalensis* JF Gmel. (100%), *Bauhinia purpurea* L. (100%), *Cymbopogon citratus* (D.C.) Stapf. (100%). A 50% concentration of *Guieria, Bauhinia,* and *Cymbopogon* species also effectively controlled the pathogen. All plant extracts inhibited *C. alatae* significantly ($P \leq 0.05$) more than the control. It was concluded that *C. alatae* can be successfully managed using any of the three strategies.

Keywords: Anthracnose, antifungal activity, *Colletoticrum alatae*, plant extracts, *Trichoderma* species, water yam

INTRODUCTION

West Africa (mainly Benin Republic, Ghana, Ivory Coast, Nigeria, and Togo) is responsible for approximately 72.39 million metric tonnes of yam tubers (95%) out of 72.6 million metric tonnes of yam tubers produced per annum globally (FAOSTAT 2018). Among root and tuber crops in sub-Saharan Africa, yam ranks first, followed by cassava and sweet potato (Amienyo and Ataga 2007; FAOSTAT 2008). Moreover, the production of water yam or *Dioscorea alata* L. (which is one of the top three most important yam species) is quite profitable; for instance, its production resulted in a gross margin and net farm income of ₦1 893 114 and ₦1 705 965, respectively in Nigeria (Nwike et al. 2017). Nigeria is the world's leading yam producer, accounting for more than 47.19 million metric tonnes of yam tubers (65%) out of 72.6 million metric tonnes of global yam tuber yield per annum (FAOSTAT 2018).

Dioscorea rotundata Poir. and *D. alata* are the most widely cultivated yam species in the world. The production of water yam is worthwhile because it does well in inferior soils compared to *D. rotundata* and actually produces more than half the quantity of yam tubers produced by white yam in such dismal situations. White yam has about 78% carbohydrates, and water yam contains about 75.65% carbohydrates (Asiedu et al. 2003; Ezeocha and Jimelukwe 2012).

Water yam has a low glycemic index and no fat, so it is often recommended for diabetic patients and those wishing to lose weight (Nestor et al. 2009; Itam et al. 2012). Furthermore, water yam exhibits ease of propagation (through the production of bulbils and reliability of sprouting), early vigor for weed suppression, and good storage ability (Asiedu et al. 2003). However, water yam's market value is lower than white yam's, particularly due to its less desirable pounding features. But water yam can be used for porridge, yam fritters, pounded yam "fufu" (i.e., yam loaves), grated water yams, and yam mixed with vegetables, especially in Nigeria. The main disadvantage of water yam is that it takes ten months to mature compared to the four to six months required to grow white yam (Oko and Famurewa 2015). This may reduce the areas where water yam can be grown.

Yam production is constrained mainly by diseases, pests, the cost of seed yams, labor, and difficulty of breeding (Sangoyomi 2004; Onuh et al. (2015). For instance, fungi, bacteria, and nematode storage rots cause the greatest yam yield losses. Ikotun (1983) reported that fungi pathogens caused 80% of storage rots of yam tubers in the West Indies and 57-77% in Nigeria. Among the diseases, *Colletotrichum gloeosporioides* causes anthracnose disease on water yam. It was reported that 95%

of the yam farmers in Ghana suffered poor yield of water yam due to anthracnose disease. The *D. alata* is the most susceptible yam species to anthracnose disease (Amusa 1997). Meanwhile, in West Africa, yam anthracnose is caused by *Colletotrichum alatae* (in *C. gloeosporioides* species complex), and it results in about 50-90% tuber yield loss (Emehute et al. 1998; Weir et al. 2012; Bhattacharjee et al. 2018; Ntui et al. 2021).

Therefore, to ensure profitable cultivation of water yam, research on control of these diseases, especially anthracnose, is highly sort after. Okigbo (2005) reported that yam anthracnose is controlled mostly with chemical fungicides, including benomyl (benlate), maneb, chlorothalonil, and mancozeb. In comparison, the combinations of copper fungicides and other synthetic fungicides like mancozeb are very effective (Johnson and Hofman 2009). But, a few years ago, Bhattacharjee et al. (2018) lamented that there are no economically feasible solutions to these disease problems, as control of diseases through chemical pesticides or cultural practices is often not convenient for small-holder resource-poor farmers. However, chemical pesticide use is still the most effective for most farmers. Onuh et al. (2015) used aqueous extracts of nine plants (fresh and dry plant materials) to control diseases in yam. Generally, fresh plant extracts showed more antimicrobial activity than dry plant extracts. Also, Okigbo (2005) reported that yam tubers inoculated with *Bacillus subtilis* showed no rot, while those inoculated with *Aspergillus niger, Botryodiploidia theobromae,* or *Penicillium oxalicum* showed considerable rot. He reported that inoculating the yam with *B. subtilis* (antagonist) and pathogens simultaneously resulted in rot symptoms. In contrast, those tubers that received *B. subtilis* a day before the fungal pathogen was inoculated resulted in completely rot-free tubers.

Okigbo and Emeka (2010) reported that *Trichoderma harzianum, Pseudomonas syringae,* and *Pseudomonas chlororaphis* reduced rot caused by *Botryodiplodia theobromae* and *Fusarium solani* by 53.5-84.0% and 59.6-87.1%, respectively. The research on managing water yam diseases has barely taken off among yam species. This research proposed some practicable botanical, chemical, and biocontrol measures appropriate to managing *C. alatae* tuber rot disease of water yam.

MATERIALS AND METHODS

Study site

The research was conducted at the Faculty of Agriculture Laboratories in Alex Ekwueme Federal University, Ndufu-Alike at Abakalik, Nigeria (6.069°N, 8.199°E). Yam (including water yam) production is a major agricultural activity in South-Eastern Nigeria, including Abakaliki.

Procedures

Sub-procedure-1: Isolation and identification of the fungi

Infected yam tubers were obtained from the main market of Abakaliki. Yam tubers were surface sterilized using 1.0% sodium hypochlorite solution for five minutes and then washed three times in sterile distilled water. Yam tubers were cut into 1 cm pieces (both infected and healthy tissues together), placed in a Petri dish containing PDA medium, and then incubated at 28°C for five days.

Trichoderma isolates were obtained from mushrooms, crop seeds, and farmland soils collected from south-eastern Nigeria and West Cameroons. Pure cultures of isolated *Trichoderma* spp. and *C. alatae* were maintained on dehydrated commercial Potato Dextrose Agar (PDA) medium.

The pure cultures were used to identify the fungi with the aid of literature on fungi morphology (Barnett and Hunter 1972).

Sub-procedure-2: Control of C. alatae *using* T. harzianum *isolates* in vitro

The experiment was laid out in Petri dishes using a completely randomized design, and each treatment was replicated three times. The treatment set consisted of *T. harzianum* isolate NSBM, *T. harzianum* isolate AIM3, *T. harzianum* isolate AIM16, *T. harzianum* isolate BGMZ4, and the control. The Control treatment was inoculated with *C. alatae* isolate alone. A 2-mm disc of the pathogen and a 2-mm disc of the biological control agent were placed at the edge of the plate according to the layout.

Sub-procedure-3: Effect of synthetic pesticides on the growth of Colletotrichum alatae in vitro

The experiment was carried out using Petri dishes. It was laid out in the laboratory using a Completely Randomized Design (CRD) with seven treatments, and each treatment was replicated three times. The treatment set included the control, mancozeb (at 100% concentration), mancozeb (at 50% concentration), Carbendazim+Mancozeb (at 100% concentration) and Carbendazim+Mancozeb (at 50% concentration), Mancozeb+Cu(I)O+Metalaxyl (at 100% concentration), Mancozeb+Cu(I)O+Metalaxyl (at 50% concentration) (Ndifon and Lum 2021; Ndifon 2022).

The Carbendazim+Mancozeb (at 50% concentration) and Mancozeb+Cu(I)O+Metalaxyl (at 50% concentration), and Mancozeb (at 50% concentration) treatments were composed by combining half the recommended rate of each pesticide.

The formulation of the synthetic fungicides was carried out thus. For Mancozeb+Cu(I)O+Metalaxyl (at 50% concentration), where there are three pesticides and mancozeb at 50%; it should be 33.33% on average of each agent. Also, at Carbendazim+Mancozeb (at 100% concentration), there are two pesticides thus 50.0% of each agenty was used, while mancozeb agent alone as a treatment at 50% concentration it was 50% of the agent.

Each treatment consisted of three levels (i.e. control 0.0, 50, and 100% concentrations) and was applied to the Petri dishes according to the layout. The in vitro rates were drawn (after obtaining the standard recommended field fungicide quantities) at 0, 50, or 100 µL per Petri dish as required.

Sub-procedure-4: Effect of plant extracts on the growth of Colletotrichum alatae in vitro

The experiment was carried out using Petri dishes. It was laid out in the laboratory using a Completely Randomized Design (CRD) with seven treatments, and each treatment was replicated three times. The treatment set included the control, *Eucalyptus globulus* L. leaves (at 50 and 100% concentrations), *Guieria senegalensis* JF Gmel. leaves (at 50 and 100% concentrations), *Bauhinia purpurea* L. leaves (at 50 and 100% concentrations), and *Cymbopogon citratus* (D.C.) Stapf. leaves (at 50 and 100% concentrations). The plant leaves utilized were weighed at 333.3 g plant leaves per L of distilled water to make 100% concentration. The 50% concentration of plant leaf extract was made by diluting one liter of 100% plant extract with one liter of distilled water. Each treatment had three levels (i.e. control 0.0, 50, and 100% concentrations). The plants were identified at National Animal Production Research Institute (NAPRI), Ahmadu Bello University, Zaria, Nigeria.

Data collection

The radius of the fungal colony was measured using a transparent rule at 24-hour intervals starting from Day 1 (i.e., 24 Hours After Inoculation (HAI)) to Day 8. The percentage inhibition of the pathogen was calculated using the equation:

$$PI = ((C - T)/C) \times 100\%$$

Where,

PI = Percentage inhibition of the growth of the fungus pathogen

C = Perpendicular* radius of the colony of the fungal pathogen in the control plate

T = Perpendicular radius of the colony of the fungal pathogen in the treated plate

*Perpendicular refers to the 'right angle' distance through the center of the dish because other radii could be obtained, especially the longest radius away from the source/front of inhibition.

Data analysis

The data were subjected to Analysis of Variance (ANOVA), and the means were separated using Student Newmann Keul's (SNK) method (as obtainable with Genstat® Discovery (Second Edition) statistical package). Descriptive statistics were used to illustrate the trends in the growth of the pathogen and its management (as obtainable with IBM Statistical Package for Social Sciences (version 25).

RESULTS AND DISCUSSION

Results

The inhibitory effect of synthetic fungicides on *C. alatae* in vitro is shown in Figure 1. The result shows that mancozeb (at 100% concentration) for the first two days had better inhibition of *C. alatae* followed by Mancozeb+Cu(I)O+Metalaxyl (at 50% concentration), Carbendazim+Mancozeb (at 50% concentration), and mancozeb (at 50% concentration). All the fungicides inhibited *C. alatae, ranging* between 8.0-76.0% inhibition.

The effect of synthetic fungicides on the growth rate of fungi is presented in Figure 2. The first day of the control shows an erratic trend, whereby some synthetic fungicides did not inhibit the pathogen more than the control. However, synthetic fungicides showed better inhibition of C. alatae than the control during the second and third growth intervals. The trend was virtually the same in the fourth growth interval, except that mancozeb (at 50% concentration) was less efficient at this stage. Based on the means separation using SNK, Carbendazim+Mancozeb (at 50% concentration), Mancozeb+Cu(I)O+Metalaxyl (at 100% concentration), mancozeb (at 100% concentration) were significantly ($P \leq 0.05$) better than all the other treatments at inhibition of the pathogen. Mancozeb (at 50% concentration) was significantly superior to Mancozeb+Cu(I)O+Metalaxyl (at 50% concentration), Carbendazim+Mancozeb (at 100% concentration), and the control.

The inhibitory effect of four *Trichoderma* isolates on *C. alatae* in vitro is shown in Figure 3. It was observed that *T. harzianum* isolate BGMZ4 was more consistent and stable in inhibiting *C. alatae*. On the first day, *T. harzianum* AIM 3 was the best isolate, while on the second day, *T. harzianum* NSBM was the best isolate, and isolate AIM 16 was the best isolate on Day 3. The results of using isolates of *T. harzianum* against *C. alatae* ranged between 16-52% inhibition. Results revealed that *T. harzianum* isolate AIM3 could not inhibit the pathogen better than the control during the first growth interval. At the same time, *T. harzianum* isolate NSBM also could not inhibit the pathogen sufficiently. During the third and fourth growth intervals (for all the isolates), the bio-control agents could inhibit the pathogen more than the Control (Figure 4).

The means separation shows that *T. harzianum* AIM16 exhibited significantly ($P \leq 0.05$) better in controlling the pathogen, followed by *T. harzianum* NSBM, *T. harzianum* BGMZ4, and *T. harzianum* AIM3. The inhibitory effect of plant extracts on *C. alatae* in vitro is shown in Figure 5. The result shows that *E. globulus* (at 50 and 100% concentration) followed by *G. senegalensis* (at 100% concentration) caused better inhibition of *C. alatae* compared to the other treatments. Generally, plant extracts showed acceptable inhibition of *C. alatae*, ranging between 8.0-96.0%. All plant extracts showed inhibitory efficacy against *C. alatae* compared to the control.

The effect of plant extracts on the growth rate of *C. alatae* is presented in Figure 6. The *E. globulus* (at 50 and 100% concentrations) effectively reduced the growth rate of *C. alatae*, followed by *G. senegalensis* (at 100% concentration). The means separation shows that the extract of *E. globulus* 100% was significantly different ($P \leq 0.05$) from the other treatments and showed the best inhibitory potential, followed by *E. globulus* (at 50% concentration), 100% concentrations of *G. senegalensis*, *B. purpurea*, and *C. citratus*. However, all plant extracts (at 50 and 100% concentrations) were significantly different ($P \leq 0.05$) compared to the control.

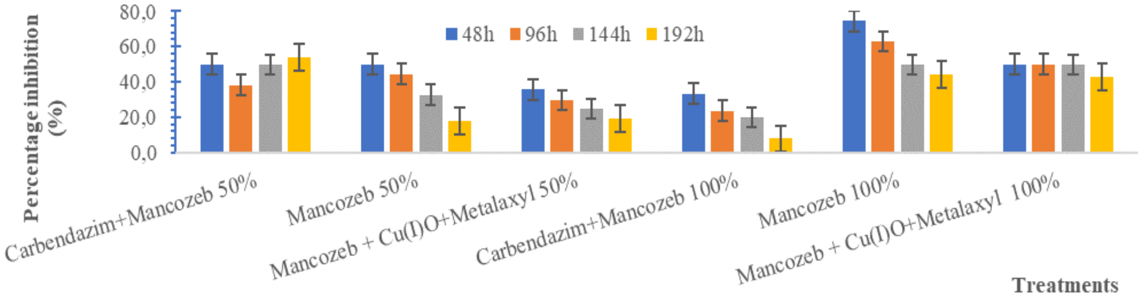

Figure 1. The inhibitory effect of synthetic fungicides on *Colletotrichum alatae*

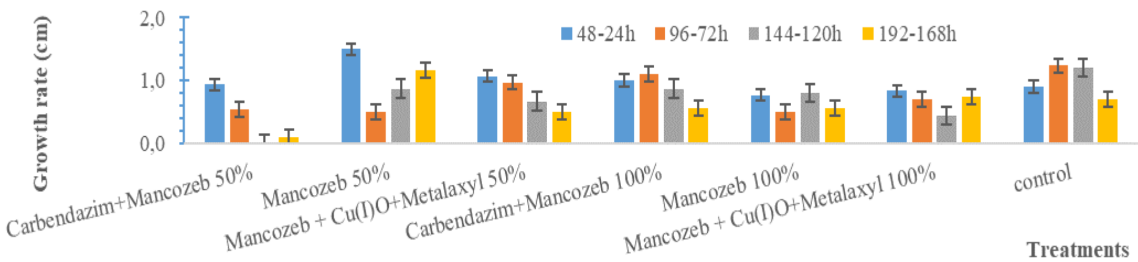

Figure 2. The effect of synthetic fungicides on the growth rate of *Colletotrichum alatae* in vitro

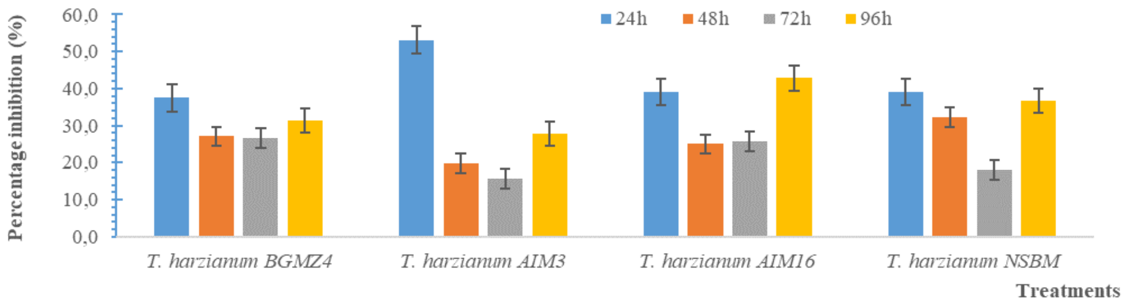

Figure 3. The inhibitory effect of *Trichoderma* isolates on *Colletotrichum alatae* in vitro

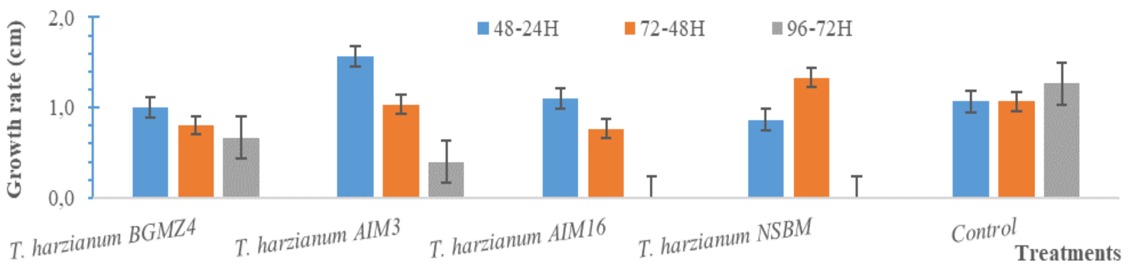

Figure 4. The effect of *Trichoderma* isolates on the growth rate of *Colletotrichum alatae* in vitro

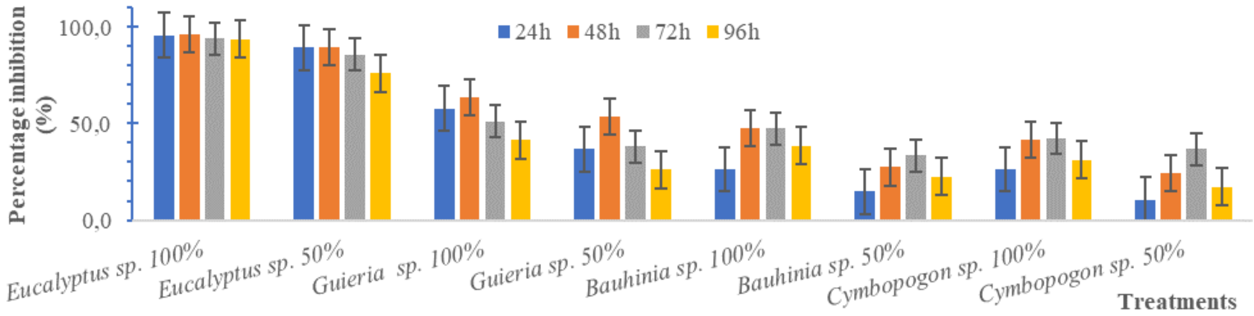

Figure 5. The inhibitory effect of plant extracts on the mycelia of *Colletotrichum alatae* in vitro

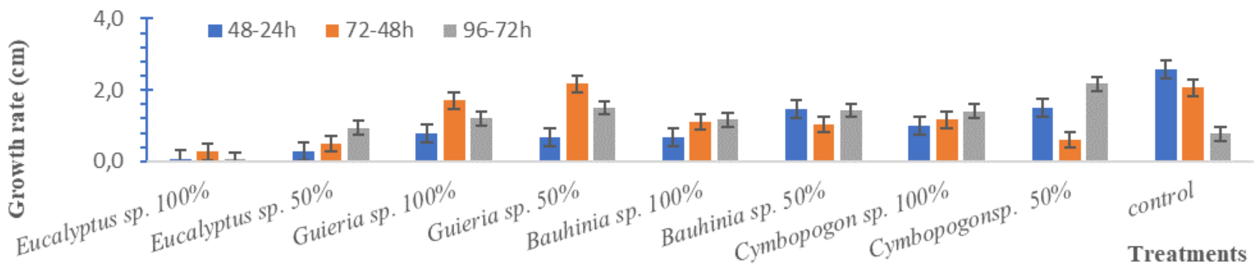

Figure 6. The effect of plant extracts on the growth rate of *Colletotrichum alatae* in vitro.

Discussion

Globally the production of *D. alata* is quite profitable, especially in Nigeria, the world's leading producer of yams (65% of 72.6 million metric tonnes of yam tubers produced per annum globally). Crop production is hampered by pests and diseases, including anthracnose in yam caused by *C. alatae*. The results of the sub-trial on the use of low rates of synthetic pesticides revealed that they could reduce the growth of *C. alatae*, which is in line with the findings of Ndifon and Lum (2021) and Ndifon (2022). Mancozeb alone showed better control potential than other fungicide treatments; this corroborated the findings of Ndifon and Lum (2021). Furthermore, Ndifon (2022) observed that the inhibition level of A. niger and *Athelia rolfsii* was more when mancozeb was applied than Mancozeb+Cu(I)O+Metalaxyl.

The result demonstrated that applying biocontrol agents against *C. alatae* was effective. These results followed the findings of Ndifon (2022), who successfully inhibited pathogenic *A. rolfsii* using *Trichoderma* and *Cladosporium* spp. Okigbo and Emeka (2010) also reported that biocontrol agents (such as *Trichoderma* and *Pseudomonas* spp.) showed promising potential against fungal pathogens.

In this present experiment, all four plant extracts (*E. globulus*, *G. senegalensis*, *B. purpurea*, and *C. citratus*) effectively inhibited the growth of *C. alatae*. Among them, *Eucalyptus* sp. plant extract was very effective against the growth of *C. alatae*. This finding is consistent with the results of Ndifon and Lum (2021), who reported that leaf extracts (i.e., *E. globulus*, *Melaleuca cajuputi* Powell, *Andrographis paniculata* (Burm.fil.) Nees, and *Azadirachta indica* A. Juss.) and the extract of shoots (i.e., *Euphorbia hirta* L.) inhibited the growth of *A. niger* significantly; Onuh et al. (2015) also successfully utilized plant extracts to control pathogenic fungi. Dania et al. (2014) reported that (in vitro and in vivo*)* plant extracts of *Oryza sativa* L. and *Quercus phillyraeoides* A. Gray were able to control *Lasiodiplodia theobromae*, *A. niger*, *Rhizoctonia solani*, *P. oxalicum*, *Sclerotium rolfsii*, and *Fusarium oxysporum* associated with white yam.

The results of the present study concluded that *C. alatae* was successfully inhibited in vitro using the four *Trichoderma* isolates (AIM 3, AIM 16, BGMZ4, and NSBM). The *C. alatae* was also effectively inhibited using synthetic fungicides like Mancozeb, Carbendazim+ Mancozeb, and Mancozeb+Copper(I) oxide+Metalaxyl. All the plant extracts (*E. globulus*, *G. senegalensis*, *B. purpurea*, and *C. citratus*) effectively controlled *C. alatae*. Thus the selected fungicides, plant extracts, and biocontrol agents could control the growth of *C. alatae*. Further research needs to be carried out to determine the effects of these control strategies in vivo.

REFERENCES

Amienyo CA, Ataga AE. 2007. Use of indigenous plant extracts for the protection of mechanically injured sweet potato (*Ipomoea batatas* (L.) Lam) tubers. Sci Res Essay 2 (5): 167-170.

Amusa NA. 1997. Fungi associated with anthracnose symptoms of yam (*Dioscorea* spp.) in south-west Nigeria and their roles in disease severity. Crop Res 13: 177-183.

Asiedu R, Mignouna H, Odu B, Hughes J.A. 2003. Yam breeding. In: Hughes JA, Odu BO (eds). Plant Virology in Sub-Sahara Africa. Ibadan, Nigeria.

Barnett HL, Hunter BB. 1972. Illustrated Genera of Imperfect Fungi. 3rd Edition, Burgess Publishing Co., Minneapolis.

Bhattacharjee R, Nwadili CO, Saski CA, Paterne A, Scheffler BE, Augusto J, Lopez-Montes A, Onyeka JT, Kumar PL, Bandyopadhyay R. 2018. An EST-SSR based genetic linkage map and identification of QTLs for anthracnose disease resistance in water yam (*Dioscorea alata* L.). PLoS ONE 13 (10): e0197717. DOI: 10.1371/journal.pone.0197717.

Dania V, Fadina O, Ayodele M, Kumar PL. 2014. Efficacy of *Oryza sativa* husk and *Quercus phillyraeoides* extracts for the in vitro and in vivo control of fungal rot disease of white yam (*Dioscorea rotundata* Poir). SpringerPlus 3 (1): 711. DOI: 10.1186/2193-1801-3-711.

Emehute JKU, Ikotun T, Nwauzor EC, Nwokocha HN. 1998. Crop protection. In: Orkwor GC, Asiedu R, Ekanayake IJ (eds). Food Yams: Advances in Research. Ibadan, Nigeria, IITA/NRCRI.

Ezeocha VC, Jimelukwe PC. 2012. The Impact of cooking on the proximate composition and anti-nutritional factors of water yam (*Dioscorea alata*). J Stored Prod Postharvest Res 3: 172-176. DOI: 10.5897/JSPPR12.031.

FAOSTAT. 2008. Food and Agriculture Organization Database on Agriculture. http://agricor.ac.uk/subject.listing/310.html

FAOSTAT. 2018. Yam production in Nigeria. Food and Agricultural Organization of the United Nations. Rome, Italy. www.fao.org. http://scholarsresearchlibrary.com/archive.html

Ikotun T. 1983. Post-harvest microbial rot of yams in Nigeria. Agric Mech Asia Afr Lat Ame 16 (1): 55-58.

Itam EH, Itam AH, Odey MO, Ejemot NR, Asenye MF, Ezike NN. 2012. Effect of processing method on the glycemic index of some carbohydrate staples (*Manihot esculanta*, *Ipomoea batata* and *Dioscorea rotundata*) in both normal and diabetic subjects. Ann Biol Res 3: 5507-5510.

Johnson GI, Hofman PJ. 2009. Postharvest technology and quarantine treatments. In: Litz RE (eds). The Mango: Botany, Production and Uses, 2nd Edition. CABI, Wallingford, UK. DOI: 10.1079/9781845934897.0529.

Ndifon EM, Lum AF. 2021. Assessment of white yam tuber rot disease and in vitro management of *Aspergillus niger* in Ebonyi State, Nigeria. Intl J Biosci 19 (4): 32-40. DOI: 10.12692/ijb/19.4.32-40.

Ndifon EM. 2022. Inhibitory efficacy of microbial, botanical and synthetic fungicides against *Athelia rolfsii (Sclerotium* stem rot) of groundnut and Bambara groundnut. Trop Agric Res Ext 25 (1): 9-17. DOI: 10.4038/tare.v25i1.5563.

Nestor KK, Gnomblesson GT, Jacko RFA, Massara CC, Georges NA. 2009. Influence of the variety and cooking method on glycemic index of yam. Pak J Nutr 8: 993-999. DOI: 10.3923/pjn.2009.993.999.

Ntui VO, Uyoh EA, Ita EE, Markson AA, Tripathi JN, Okon NI, Akpan MO, Phillip JO, Brisibe EA, Ene-Obong EE, Tripathi L. 2021. Strategies to combat the problem of yam anthracnose disease: Status and prospects. Mol Plant Path 22 (10): 1302-1314. DOI: 10.1111/mpp.13107.

Nwike MC, OKoli TO, Ugwumba COA. 2017. Economics of water yam (*Dioscorea alata*) production in south eastern Nigeria. IOSR J Agric Vet Sci 10(7 Ver. II):13-18. DOI: 10.9790/2380-1007021318.

Okigbo RN, Emeka AN. 2010. Biological control of rot-inducing fungi of water yam (*Dioscorea alata*) with *Trichoderma harzianum*, *Pseudomonas syringae* and *Pseudomonas*. *Chlororaphi*. J Stored Post Res 1 (2): 18-23.

Okigbo RN. 2005. Biological control of postharvest fungal rot of yam (*Dioscorea* spp.) with *Bacillus subtilis*. Mycopathologia 159: 307-314. DOI: 10.1007/s11046-004-2454-8.

Oko AO, Famurewa AC. 2015. Estimation of nutritional and starch characteristics of *Dioscorea alata* (water yam) varieties commonly cultivated in the South-Eastern Nigeria. Brit J Appl Sci Tech 6: 145-152. DOI: 10.9734/BJAST/2015/14095.

Onuh JO, Shiriki D, Ubwa ST, Shambe T. 2015. Isolation of six microorganisms from rotten *Dioscorea alata* (water yam), and antimicrobial sensitivity test with nine plant extracts. Food Nutri Sci 6:1381-1394. DOI: 10.4236/fns.2015.615144.

Sangoyomi TE. 2004. Post-Harvest Fungal Deterioration of Yam (*Dioscorea* spp.) and Its Control. [Ph.D. Dissertation]. University of Ibadan, Ibadan. [Nigerian]

Weir BS, Johnston PR, Damm U. 2012. The *Colletotrichum gloeosporioides* species complex. Stud Mycol 73: 115-180. DOI: 10.3114/sim0011.