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From The Editor;

Dear Readers and Authors,

As “International Journal of Science Letters (IJSL)”, we are pleased and honored to present the last issue of 2022. IJSL, is an international double peer-reviewed open access academic journal published on the basis of research- development and code of practice.

The aims of this journal are to contribute in theoretical and practical applications in relevant researchers of Life Sciences, Biology, Biotechnology, Bioengineering, Agricultural Sciences, Food Biotechnology and Genetics institutions and organizations in Turkey, and to publish solution based papers depending on the principle of impartiality and scientific ethics principles, focusing on innovative and added value work, discussing the current and future.

With these thoughts, we are especially thankful to academicians honoring with the articles, valuable scientists involved in editorial boards and reviewers for their contributions to the evaluation processes with through their opinions/ideas/contributions/criticisms in this issue of International Journal of Science Letters.

26.08.2022

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Research Article

Determination of the antiproliferative effect of *Folliculj sennea* used as a laxative on CCD-18Co cell line and proliferative effect on DLD-1 and HT-29 cancer cell lines

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Abstract

The most prominent feature of malnutrition that increases colon cancer is the use of laxatives. Most community-dwelling individuals self-manage the condition of bowel-related diseases and do not seek medical advice. Self-management often involves the use of laxative products that can be purchased over the counter from pharmacies and elsewhere. According to the research, most of those who use herbal products do not get enough information about the products they use, and the most important problem is that they do not inform their health consultants (doctors, pharmacists, dietitians, nurses, etc.) about the product they use. Individuals get information about the product they use from transfers such as uncontrolled media channels on the internet, and they reach the product easily. Long-term use of laxatives is predicted to impair healthy colonic function, produce laxative dependence, and damage the enteric nervous system and/or intestinal smooth muscle. It manages colon motility and may increase the risk of other types of cancer, especially colon cancer. In our study, the antiproliferative effect of *Folliculj sennae* plant, which is commonly used as a laxative, known as fasting herb, horseradish herb, and camel eye herb and contains an anthracoid laxative, on CCD-18Co (healthy colon epithelium) cell line and DLD-1 (colon cancer) and HT-29 (colorectal cancer) cancer cell lines, on the other hand, aimed to determine its proliferative effect by MTT analysis.

Article History

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Keywords

Folliculj sennae,
Laxative,
MTT,
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1. Introduction

Colon cancer is the third most common type of cancer worldwide. Colon cancer risk factors that are especially related to lifestyle are smoking, alcohol consumption, obesity, physical inactivity, and some malnutrition and diet factors. It is estimated that approximately 70% of colon cancer cases can be prevented by following a healthy lifestyle, and basic lifestyle factors that can be changed with medical nutrition therapy are at the forefront (Jakszyn et al.,

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2020). Adiposity and lack of physical activity are two other major factors that can contribute to chronic inflammation, and they are highly correlated with diet and are also considered important causes of colon cancer (Clinton et al., 2020). Due to the lack of standardization in the nutrition of individuals, the effects of malnutrition may increase the risk of colon cancer and may hurt the healthy colon epithelium. The most prominent feature of malnutrition that increases colon cancer is the use of laxatives. Most community-dwelling individuals self-manage the condition of bowel-related diseases and do not seek medical advice. Self-management often involves the use of laxative products that can be purchased over the counter from pharmacies and elsewhere. Laxatives have the properties of accelerating or stimulating defecation and are used for many purposes in the community, especially for constipation management. Laxatives generally show this effect by three different mechanisms. Mechanism pathways: (i) enhancing fluid retention through hydrophilic or osmotic mechanisms; (ii) reducing the net absorption of fluid through effects on small and large intestinal fluid and electrolyte transport; or (iii) segmentation is to alter motility by inhibiting (non-impulsive) contractions or by stimulating repulsive contractions (Werth et al., 2020). When looking at the products used as laxatives, herbal products come to the fore. The most important problem in the treatment with herbal products is the use of plants without adequate clinical research as if they are medicine because they are natural, and the unawareness that serious problems may arise as a result of the interaction of herbal products with medicines, among themselves and with food. According to the research, most of those who use herbal products do not get enough information about the products they use, and the most important problem is that they do not inform their health consultants (doctors, pharmacists, dietitians, nurses, etc.) about the product they use. Individuals get information about the product they use from transfers, uncontrolled media channels, and the internet, and they reach the product easily (Uzun et al., 2014). Long-term use of laxatives is predicted to impair healthy colonic function, produce laxative dependence, and damage the enteric nervous system and/or intestinal smooth muscle. It manages colon motility and may increase the risk of other types of cancer, especially colon cancer. In our study, the antiproliferative effect of the *Folliculj sennae* plant, which is commonly used as a laxative and known as fasting grass, horseradish herb, and camel eye herb and contains an anthracoid laxative, on CCD-18Co (healthy colon epithelium) cell line and DLD-1 (colon cancer) and HT- 29 (colorectal cancer) cancer cell lines, on the other hand, aimed to determine its proliferative effect by MTT analysis.

2. Materials and Methods

2.1. Test Item

1 mg/ml main stock was prepared by dissolving the *Folliculj sennae* plant in distilled water in the experimental study. Other concentrations (100; 50; 25; 12,5; 6,25; 3,125 and 1,56 mg/ml) were prepared by serial dilutions of the 1000 mg/ml master stock concentration.

2.2. Cell Culture

In cell culture studies, necessary media and an appropriate environment were provided for the cells to live and reproduce *in vitro.* The medium requirement differs according to the type of cells and their adaptability. Our study used three different media (EMEM, McCOY, DMEM) for three different cell lines (CCD18-Co, HT-29, DLD-1). The media of the cells were kept in an incubator at 37°C, 95% humidity and 5% CO_2 were changed twice a week and the development of the cells was monitored.

2.3. MTT Analysis

When the cells were confluent, they were first washed with PBS (phosphate buffered saline), removed from the flasks using trypsin-EDTA, and the cells taken into the falcon during passage were centrifuged at 16 000 rpm for 10 minutes. 1000 µL of the medium was added to the pellet under the falcon, and the pellet was dissolved, 10 µL of the medium-cell mixture was added to a 0.2 ml tube, and 10 µL of Trypan-blue dye was added. 10 µL of the mixture was taken and spread between the Thoma slide and coverslip. Cells in 16 squares on the Thoma slide were counted using a light microscope. The number of cells was determined according to the formula A x 2 x104. It was taken into 96-well plates and used in MTT analysis. The purpose of using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) method; is based on the conversion into formazan crystals by living cells, which determines mitochondrial activity. The cells were seeded onto the plates with the help of a multi-pipe so that the calculated amount of cells was poured into each well of the 96-well plates. The seeded cells were kept in the incubator for 24 hours to adhere to the plate surface. Other concentrations to be studied (100; 50; 25; 12,5; 6,25; 3,125 and 1.56 mg/ml) were prepared with serial dilutions of the master stock concentration prepared with *Folliculj*

sennae used in the experimental study, and *Folliculj sennae* at the concentrations prepared for each cell line was added to the plate in triplicate. . Negative control (cell control), positive control (mitomycin-C) and 1/1000 DMSO concentrations were added to the plate in triplicate and left in the incubator for 24 hours. Since MTT dye is a light-affected dye, 5 mg was weighed for 1 plate in the dark, 1 mL of PBS (phosphate buffered saline) was added to it and 8 mL of medium was added and dissolved by vortexing. The prepared MTT solution was inoculated on the plates and the plates covered with aluminum foil were kept in the incubator for 2-4 hours. At the end of the period, the MTT solution was aspirated and 100 µL of DMSO (100%) was added to each well to stop the reaction. After the plate was kept in the dark for 10 minutes, absorbance values were read spectrophotometrically at a wavelength of 570 nm. The effect of the MTT method on cell density in working cell lines was determined with the concentrations applied with the help of the Microsoft Excel program, and the 50% inhibitory concentration value was calculated.

3. Results

The CCD-18Co cell line; it showed the best effect on the cell viability of the *Folliculj sennae* plant at 100 mg/ml concentration. The % viability activities in the CCD-18Co cell line were determined between 51 and 83% (Table 1) (Figure 1). Therefore, it is thought to have antiproliferative activity in the CCD-18Co cell line.

Table 1. MTT absorbance measurements in CCD-18Co cell line

		% Viability
Cell Control	1,0306	100,0000
Mitomycin-C	0,4105	39,8312
100	0,5289	51,3196
50	0,5884	57,0930
25	0,6257	60,7122
12,5	0,7365	71,4632
6,25	0,6368	61,7892
3,125	0,8625	83,6891
1,5625	0,7325	71,0751

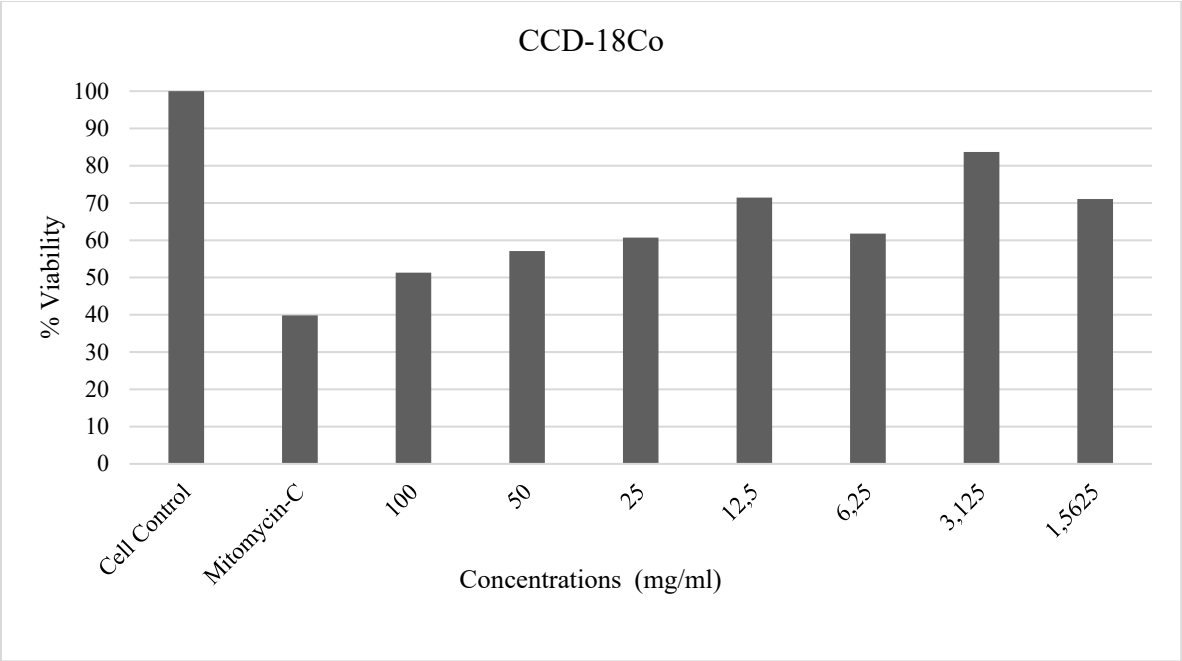

Figure 1. Comparison of percent viability of concentrations in CCD-18Co cell line

The effect of the *Folliculj sennae* plant on cell density was read with a spectrophotometer using the MTT method, the percent viability curve was determined with the help of the Microsoft Excel program, and the 50% inhibitory concentration value (IC_{50}) was calculated with a bar graph and a logarithmic slope line was drawn. The 50% inhibitory concentration (IC_{50}) value from the logarithmic slope line was determined as 67,4 mg/ml.

The DLD-1 cell line; It showed a high effect on the cell viability of *Folliculj sennae* at all concentrations (Table 2) (Figure 2). Therefore, it is thought to have proliferative activity in the DLD-1 cell line.

Table 2. MTT absorbance measurements in DLD-1 cell line

		% Viability
Cell Control	1,4060	100,0000
Mitomycin-C	0,6499	46,2233
100	4,4956	319,7440
50	4,5002	320,0711
25	6,0000	426,7425
12,5	5,3000	376,9559
6,25	6,0000	426,7425
3,125	6,0000	426,7425
1,5625	6,0000	426,7425

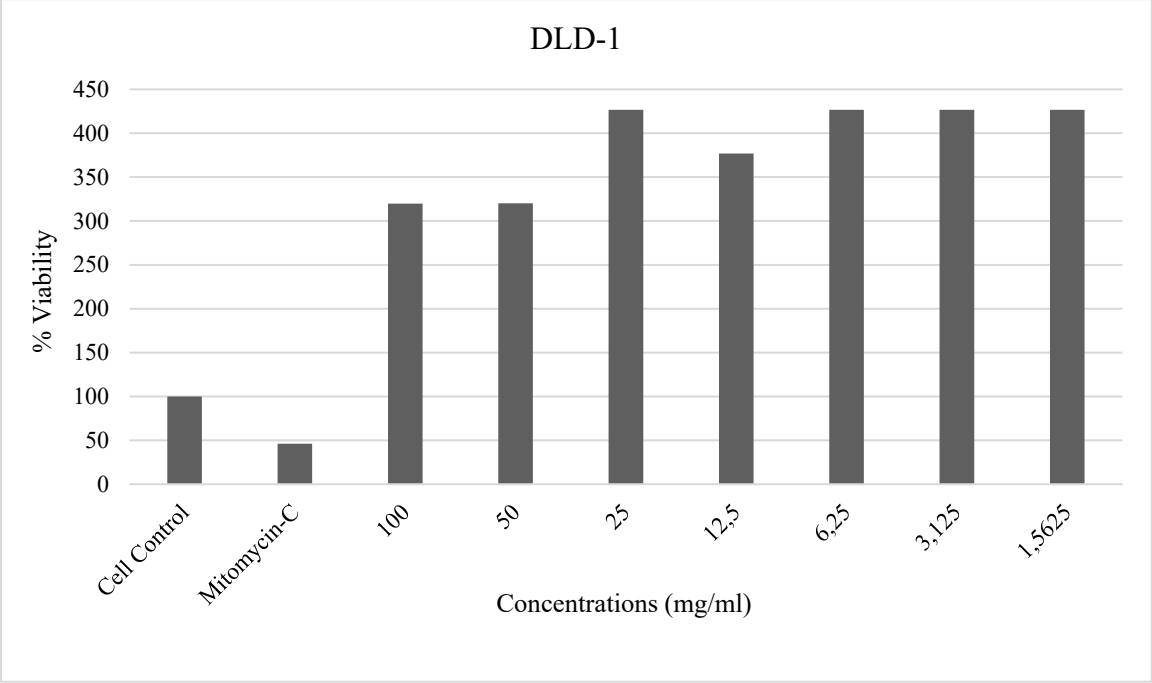

Figure 2. Comparison of percent viability of concentrations in DLD-1 cell line

Since the effect of the *Folliculj sennae* plant on cell density was high at all concentrations by the MTT method, the 50% inhibitory concentration (IC_{50}) value could not be calculated from the logarithmic slope line.

The HT-29 cell line; It showed a high effect on the cell viability of *Folliculj sennae* at all concentrations (Table 3) (Figure 3). Therefore, it is thought to have proliferative activity in the HT-29 cell line.

Table 3. MTT absorbance measurements in HT-29 cell line

		% Viability
Cell Control	1,4060	100,0000
Mitomycin-C	0,8241	58,6131
100	6,0000	426,7425
50	5,5000	391,1807
25	5,1000	362,7312
12,5	5,3000	376,9559
6,25	4,6000	327,1693
3,125	5,1000	362,7312
1,5625	4,2000	298,7198

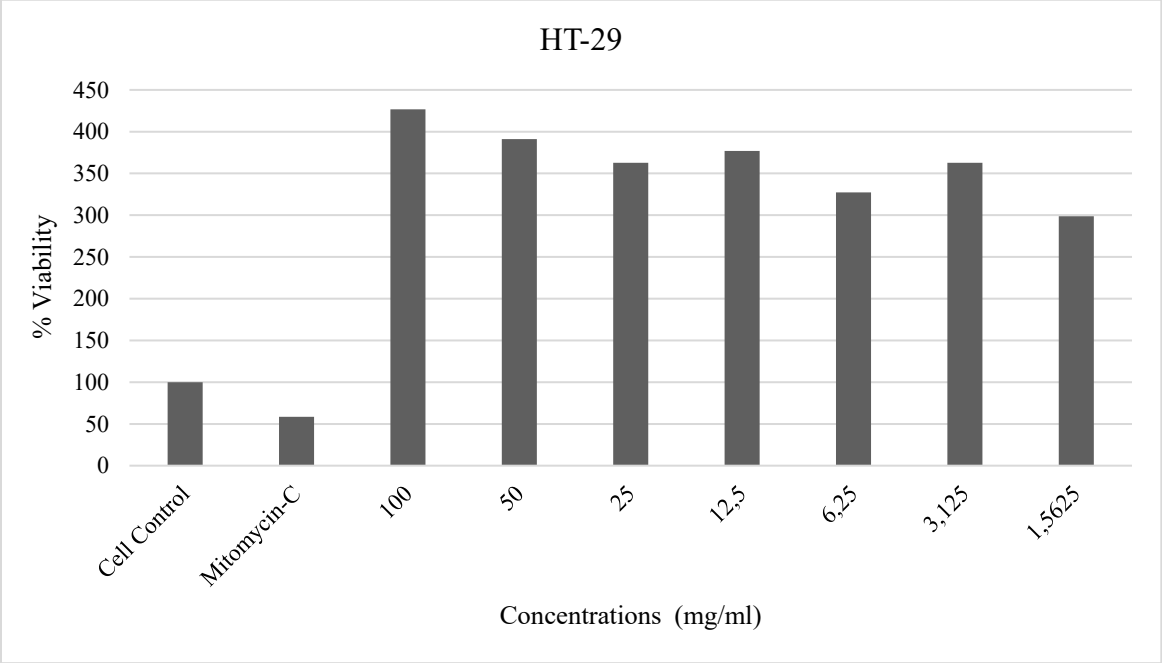

Figure 3. Comparison of percent viability of concentrations in HT-29 cell line

Since the effect of the *Folliculj sennae* plant on cell density was high at all concentrations by the MTT method, the 50% inhibitory concentration (IC_{50}) value could not be calculated from the logarithmic slope line.

4. Discussion

The purpose of our experimental study; the aim in this study is to investigate the proliferative effects of the *Folliculj sennae* plant, which is unconsciously used as a laxative against colon cancer, which is the most common type of cancer worldwide, in colon cancer cell lines, and antiproliferative effects on healthy colon epithelium. In 2018, Citronberg et al. examined the relationship between non-fiber laxative use and fiber-based laxative use and colorectal cancer risk in a multisite International Colon Cancer Family Registry cohort study of 4025 controls. Epidemiological risk factor questionnaires were administered to all participants and exposures were determined approximately 1 year before diagnosis for cases and over a comparable period for controls. Known and suspected risk factors for colorectal cancer have been identified, including regular use of laxatives, defined as a laxative intake for more than one month at least twice a week. People who reported that they regularly used non-fiber-based laxatives were at a significantly higher risk for colorectal cancer than those who reported that they never used laxatives (Citronberg et al., 2018). Among 65,838 women without cancer, the ACS guidelines were associated with a 61% lower risk of colorectal cancer-specific mortality. Among women who survived cancer in 2017, women who had diets consistent with American Institute of Cancer Research guidelines had a 20% lower risk of death over the study period. In 1 of the other studies examining the post-diagnosis diet quality score, women who had diets consistent with the Healthy Eating Index after breast cancer had a 26% lower risk of death over the study period (George et al., 2014). Emodin is an anthraquinone stimulant laxative and is used to treat constipation, and to use it effectively in the treatment of constipation, understanding its mode of action and potential drug targets is of paramount importance. Emodin has provided convincing evidence that it is associated with increased expression of AQP3 by upregulating the PKA/p-CREB signaling pathway by increasing the production and gene expression of AQP3 in the HT-29 cancer cell line (Zheng et al., 2014). Sennoside A is a representative of anthraquinone laxatives and gut microbes may play an important role in the purgative mechanism of sennoside A. Sennoside A caused an increase in the level of epithelial cell proliferation near the tumor tissue. It leads to a possible

malignant change in the proliferation of epithelial cells, suggesting that Sennoside A has a tumor-promoting effect in colitis-associated colonic disease. They observed that sennoside A can disrupt the intestinal mucosal barrier by disrupting the homeostasis of intestinal bacteria and causing the production of intestinal epithelial inflammation, thus promoting the development of colon cancer. Beginning with the key initial link in the occurrence of colon cancer, microbiota-mediated mechanisms linking long-term anthraquinone laxative intake to gastrointestinal inflammation and cancer progression have not been identified. Anthraquinone laxatives and laxative botanicals anthraquinone-containing compounds are frequently used clinically, but the use of anthraquinones for the possible risk development of colorectal neoplasms has long been controversial. Studies show that anthraquinone damages the structure of the colon epithelial tissue. It shows that long-term anthraquinone use has a certain correlation with the development of colon cancer. However, the limited evidence cannot favorably interpret the potential adverse effects of anthraquinone laxatives (Wei et al., 2020). Pseudomelanosis coli is associated with the use of anthraquinone laxatives. This finding has been replicated in animal studies. The genotoxic potential of anthraquinone laxatives has been documented in many in vitro studies. Tanaka et al. observed that rats exposed to a diet containing anthraquinone developed colon carcinoma after 480 days (Tanaka et al., 1990). Several human studies reported a positive relationship between laxative use and colorectal cancer; A prospective case-control study in Germany investigated the risk of anthraquinone laxatives in the development of colorectal adenoma and colorectal carcinoma compared with patients without colorectal neoplasms. They reported no increased risk for the development of colorectal adenomas or colorectal carcinomas. By logistic regression analysis, the odds ratio for colorectal adenomas was calculated to be 0.84, even after adjusting for anthraquinone laxative use time, age, sex, and blood in the stool (Nusko et al., 2000).

5. Conclusion

The cause-effect relationship between unconscious laxative use and colon cancer remains unclear, although several studies have investigated the role of the gut microbiota in the pathogenesis of colorectal cancer. Still, laxatives have been associated with increased tumor detection since first linking high fiber intake to be protective against the development of colorectal carcinoma. In addition to laxative use, various environmental, genetic, and lifestyle factors play a role in the pathogenesis of colorectal cancer. The risk of these factors developing colorectal cancer is unknown. Considering the strong side effects caused by

unconsciously using laxatives by evaluating the findings, the effect of the *Folliculj sennae* plant, which we have witnessed positive effects on DLD-1 and HT-29 cancer cell lines and negative effects on CCD-18Co cell line, on human health should be investigated with large-scale studies. It is thought that the result of our study will make an important contribution to raising the awareness of patients about laxatives by health professionals.

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Research Article

Screening for drought tolerance in cowpea at the flowering stage

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Abstract

Drought is one of the major threats to cowpea productivity in tropical countries, and understanding its impacts is germane in ensuring food security in a global context. The present study was established to screen some accessions of cowpea for drought tolerance at the flowering stage in pots under the controlled conditions of a screen house. High significant differences were observed among accessions for wilting and recovery traits, stomatal conductance, relative water content (RWC), terminal leaflet length (TLL) and width (TLW), stem girth, and yield parameters under drought stress. In addition, drought stress caused a significant reduction in morphological traits and RWC between the initial and the final values. Based on cluster and Principal Component Analysis (PCA), accessions were separated into different classes of tolerance. Direct selection for wilting traits, stomatal conductance, morphological traits, and recovery parameters showing high heritability (≥ 60%), GAM (≥ 20%), and PCA (≥ 0.4) will be effective. Hence, four major classes of tolerance were determined: AC03, AC08, and AC10 were highly susceptible. AC01 and AC04 were moderately susceptible. AC06, AC07, and AC09 were moderately tolerant, while AC02 and AC05 were the highly tolerant accessions. The moderately tolerant and the highly tolerant accessions showed a combination of superior resistance to wilting, superior recovery rates, and superior yield attributes. They also showed lower stomatal conductance, higher RWC, and low reduction of RWC, TLW, and stem girth under drought stress compared to the susceptible ones.

Article History

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Keywords

Accessions,
Biplot,
Heritability,
Productivity,
Recovery,
Selection

1. Introduction

Drought is one of the greatest threats to crop productivity, including cowpea (*Vigna unguiculata* L. Walp) in the tropical and subtropical countries of the world. Understanding the impacts of

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drought on crop productivity is germane to ensuring food security in a global context (Leng & Hall, 2019). Drought refers to a condition of sustained moisture deficit in soil capable to hinder crop growth and development with a significant reduction in yield (Ajayi et al., 2018). Drought is one of the main implications of climate change (Santos et al., 2020; Choudhary et al., 2021; Cirillo et al., 2021; Onyemaobi et al., 2021; Shanmugam et al., 2021). It is significant to agricultural production, with a disastrous effect projected for many plant species worldwide (Tebeje et al., 2017; Cirillo et al., 2021; Wasae, 2021). Crops under drought stress resist evapotranspiration by stomata closure, but as a result, carbon absorption is cut down during photosynthesis, thereby stunting productivity (Gomes et al., 2020; Adusei et al., 2021). The continuous growth of the global population (Khatun et al., 2021), foreseen to climax at nine billion people by the year 2050 (Santos et al., 2020), with Nigeria contributing significantly to it (Ajayi et al., 2022), is expected to double the global food demand by 2050 (Gomes et al., 2020). In the global context, vis-a-vis attaining food security, cowpea has been recognized as one of the key crops in realizing such a feat. Specifically, both economic, social, and environmental dimensions of the sustainable development goals of the Food Agriculture Organization (FAO) recognize the potential of cowpea in meeting food security (Nunes et al., 2022). Consequently, there is a need for an adequate understanding of the source of genotypic differences for drought tolerance among available cowpea varieties, especially in Nigeria that can be deployed to breeding for drought tolerance to improve its productivity.

In the tropical and subtropical regions of the world, for ages, one of the major sources of protein especially among the poor is cowpea (Ezin et al., 2021). This is because, compared to animal protein, it is cheaper; and has been found to contain around 25% protein in its seeds (Ajayi et al., 2018). Aside from this, it is rich in vitamins, minerals, fiber, and carbohydrates. It is useful in an intercropping system for being able to fix atmospheric nitrogen in the soil for soil replenishment (Santos et al., 2020). It is also one of the major sources of protein for animals as the vegetative parts are fed to cattle (Nunes et al., 2022). Cowpea thrives well in the tropical ecosystem; it has a higher tolerance to a couple of abiotic stresses including heat, drought, and soil acidity compared to cereals, however, a high level of genotypic differences exists among available varieties regarding these abiotic stresses, especially as regards drought. Drought is a major threat to the crop's productivity especially during the flowering and grain filling stage (Nunes et al., 2022), despite its good attributes. Many high-yielding varieties are susceptible to drought making yield

loss of devastating magnitude a common occurrence in tropical and subtropical Africa, most especially in Nigeria. It is, therefore, necessary to gather adequate information regarding the genetic differences among available varieties for drought tolerance to deploy such information in the plant breeding program of the crop. Presently, Nigeria accounts for the highest production of cowpea in the world, contributing about 2.61 metric tons per annum accounting for about 36% of the global production of the crop (Ajayi et al., 2022).

Drought at the flowering stage of crops as the consequence of heavy reliance of the agricultural systems in tropical Africa on rain-fed agriculture is devastating to yield. Rain-fed agriculture is characterized by uneven distribution and uncertainty at the reproductive stage, hence causing terminal drought stress with accompanying reduction in yield (Lemma et al., 2021), which may climax between 70 and 80% (Harshani & Fernando, 2021; Wang et al., 2021). The devastating effects of drought stress during the reproductive stage have been confirmed in several crop species including beans (Wasae, 2021), cowpea (Ezin et al., 2021; Santos et al., 2020), maize (Al-Naggar et al., 2011; Badu-Apraku et al., 2021; Khan et al., 2021), mung bean (Singh et al., 2021), rice (Garrity & Toole, 1994; Yang et al., 2019; Hussain et al., 2021), soybean (Moloi & van der Merwe, 2021), tomato (Sivakumar & Srividhya, 2016), wheat (Onyemaobi et al., 2021). Meanwhile, information regarding the mechanisms of their responses to drought during flowering is to a large scarce, making breeding for drought tolerance a difficult task (Yang et al., 2019). Therefore, screening for drought tolerance must consider the reproductive stage and also adopt simple, cheap, and non-destructive screening methods with high efficiency in identifying the level of differences as well as sources of such variations among available germplasm (Ajayi et al., 2018). Simple methods that have proven successful include stomata behavior (Nkouannessi, 2005; Agbicodo, 2009), root traits (Matsui and Singh, 2003; Santos et al., 2020), leaf rolling (Matthew, et al., 1990), leaf wilting scales, and indices (Mai-kodomo et al., 1999; Pungulani et al., 2013; Ajayi et al., 2020), plant wilting scales and percentage of wilted plants and recovery parameters under drought stress (Nkouannessi, 2005; Ajayi et al., 2018). Pot evaluation methods under controlled environments have been proven more reliable in pinpointing the level of genotypic differences for different growth stages including reproductive stage drought tolerance in crop species (Goufo et al., 2017). This is because the inducement of drought can be reliably done in a controlled environment compared to what is obtainable under field conditions (Fatokun et al., 2012; Nkomo et al., 2020; Moloi & van der Merwe, 2021).

The present study was executed in line with the following objectives: 1). to screen accessions of cowpea for drought tolerance at the flowering stage by utilizing their shoot, physiological, and yield traits. 2). to estimate the level of variability, heritability, and association of the drought-responsive traits of cowpea under drought stress at the flowering stage. 3). to confirm the consistency of the flowering stage drought tolerance of the accessions to their previous levels of tolerance at the seedling and vegetative stages.

2. Materials and Methods

2.1. Materials

The ten accessions used in the present study are presented in Table 1. These accessions had previously been screened at the seedling (Ajayi et al., 2018) and the vegetative stages (Ajayi et al., 2020) for their tolerance to drought. Pre-planting soil analysis was done; topsoil was collected, air-dried, and thoroughly mixed, sieved, and physicochemical properties were determined by spectrometry method (Table 2).

Table 1. Selected accessions of cowpea and their previous drought-tolerant statuses at the seedling and vegetative stages

S/N	Accession ID	Biological status	Seedling stage	Vegetative stage	Code
1	**TVu-199**	Breeding material	Drought tolerant	Moderately tolerant	AC01
2	**TVu-207**	Breeding material	Drought tolerant	Moderately tolerant	AC02
3	**TVu-218**	Breeding material	Highly susceptible	Highly susceptible	AC03
4	**TVu-235**	Breeding material	Drought tolerant	Moderately tolerant	AC04
5	**TVu-236**	Breeding material	Moderately tolerant	Moderately tolerant	AC05
6	**TVu-241**	Breeding material	Drought tolerant	Drought tolerant	AC06
7	**IT98K-205-8**	Unknown	Moderately tolerant	Drought tolerant	AC07
8	**IT98K-555-1**	Unknown	Highly susceptible	Highly susceptible	AC08
9	**TVu-4886**	Landrace	Moderately tolerant	Moderately tolerant	AC09
10	**TVu-9256**	Landrace	Highly susceptible	Highly susceptible	AC10

Table 2. Pre-planting properties of the topsoil used to screen the accessions of cowpea for drought tolerance at the flowering stage

S/N	Parameters	
1	pH	6.50
2	Total organic matter (%)	2.35
3	Available P (c mol/kg)	2.50
4	Total N (%)	0.36
5	H^+ (mmol)	1.05
6	Al^{3+} (mmol)	2.20
7	Na (ppm)	25.30
8	Cu (ppm)	0.90
9	Mg (ppm)	51.00
10	Pb (ppm)	0.60
11	Mn (ppm)	79.00
11	Sand (%)	78.80
12	Silt (%)	10.56
13	Clay (%)	10.64
14	Texture class	Sandy Loam

2.2. Procedures

The evaluation of the accessions was performed in pots (with three perforations each for draining excess moisture) in the screen house during the flowering stage between June and September 2016 as described by Nkouannessi (2005) with minor modifications. The seeds were planted in plastic pots filled with 7 kg of sieved topsoil with no added fertilizer. At the emergence of seedlings, plants were thinned to three (3) fairly identical plants in each pot, with five (5) pots per accession in three replicates using a Completely Randomized Design (CRD); a total of 450 plants were contained in the screen house. Each pot was watered with 500 ml of water per day till more than 80 percent of the plants in the screen house have flowered; thereafter watering was terminated for 21 days to impose drought stress. The 500 ml was predetermined according to Ogbaga et al. (2014) as the amount required to bring each pot to 100% field capacity.

2.3. Shoot wilting parameters

On days 14 and 21 of drought stress, susceptibility was scored for plants based on the 1983 Descriptors for cowpea of the International Board for Plant Genetic Resources (IBPGR). One

plant per pot was tagged for relative water content (RWC), while the two remaining plants per pot were untagged. The drought susceptibility score (DSS) on a 1 – 7 scale was used on the untagged plants, where 1 to 3 indicated low susceptibility (plant growing well with green leaves); 4 to 5 indicated medium susceptibility (a plant having most of the leaves turned yellow / or wilting), and 6 to 7 indicated high susceptibility (dead and dry plant). The mean score of plants per replicate and across the three replicates was respectively calculated. Accordingly, the percentage of wilted plants (PPW) was documented for each accession. However, the leaf wilting index (LWI) was determined for days 7, 14, and 21 under drought stress, as the ratio of leaves per plant showing wilting signs or wilted to the total number of leaves according to Pungulani et al. (2013). Accessions were ranked from 1 to 10 based on their superiority for each of the wilting traits where 1 signified the best and 10, the poorest.

2.4. Measurement of morphological parameters

Morphological traits like the terminal leaflet length (TLL) and width (TLW) of fully expanded middle terminal leafleafletsplants were determined on the initial day of imposing stress and day 7 utilizing a meter rule on two-terminal leaflets per pot per replicate. The girth of the stem of each accession per replicate was measured at 2 cm above the surface of the soil on two plants per pot using a digital vernier caliper, to the nearest millimeter at the initial day of imposed stress; 7 days, 14 days, and 21 days of the imposition of drought stress. The ranking of accessions for these traits was based on the percentage reduction in mean performance between initial and final values, where 1 signified the most superior (least reduction in mean value) and 10, the poorest (highest reduction in mean value).

2.5. Yield parameters

To the determination of the percentage pod set, twenty (20) flowers per accession per replicate were tagged on the last day of watering before the imposition of drought stress on only the matured flowers. The percentage pod set was calculated as the ratio of the number of pods formed and survived to maturity to the number of flowers tagged and expressed as a percent according to Kumar et al. (2008). The total number of pods per plant was done by counting the number of pods per plant per replicate at maturity. A mean number of pods of two plants per pot

was recorded, then the mean across replicates was determined. Pod weight was determined by selecting the ten (10) best pods per replicate after the removal of seeds. The average pod weight was determined per replicate for each accession, after which the mean pod weight for three replicates was determined. The number of seeds per pod was determined by averaging the number of seeds per pod of two plants per pot. The mean number of seeds per pod for each accession was recorded and the mean for the three replicates was determined. Seed yield per plant was determined by averaging the yield of two plants in each pot. The mean seed yield for each accession was recorded and the mean for three replicates was determined. Accessions were ranked for each trait between 1 and 10, where 1 designated the most superior (highest mean performance), and 10 designated the most inferior (lowest mean performance).

2.6. Physiological parameters

The relative water content (RWC) was done by the methods of Kumar et al. (2008). This was done for the initial, 7 days, and 14 days after the termination of watering from two young fully expanded leaflets from the top of each plant per pot per replicate. They were detached and weighed for the fresh weight (FW), afterwards, they were placed in small bags containing distilled water and kept in the refrigerator for 12 hours. Turgid weights (TW) were determined by first blotting the leaves to dryness after removing from water and weighing, as well as dry weight (DW) after drying the leaves in the oven for 48 hours at 60°C. RWC (%) was determined on each leaflet by using the formula: RWC = (FW – DW)/ (TW – DW) × 100. The average value for each replicate was determined per accession, after which the mean value of each accession was determined on the three replicates. Stomatal conductance was done only on the 14th day using a steady-state Leaf Porometer. This was done between 11.30 am and 5.00 pm utilizing surviving leaflets from among those used for TLL and TLW on only three selected plants of each accession per replicate. The average value of each parameter was determined for each accession per replicate and the mean for the three replicates was determined. The ranking of accessions for RWC was based on the percentage reduction in mean performance between initial and final values, where 1 signified the most superior (least reduction in mean value) and 10, the poorest (highest reduction in mean value). However, accession with the highest stomatal conductance was ranked 1 (best) while the one with the lowest mean performance was ranked 10 (poorest).

2.7. Determination of recovery parameters

Watering was recommenced after twenty-one (21) days of drought stress. Fourteen (14) days later, the percentage of plants that recovered in each accession was documented. Centered on this percentage, plants were ranked between 1 (highest percentage recovery) and 10 (lowest recovery percentage). Stem greenness was scored on day 14 using a scale of 1–5, where 1 indicated the recovered plant is yellow and 5 was a completely green plant. The stem re-growth was scored in three categories: 1 signified plant recovered, but with no re-growth; 3 signified plant with re-growths from auxiliary buds; and 5 signified re-growth from the shoot apexes (Pungulani et al., 2013). Accessions were ranked between 1 and 10 based on their overall mean performance for stem greenness and stem re-growth.

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed in SPSS version 20. Means were divided at a $P \leq 0.05$ level of significance using the Duncan Multiple Range Test (DMRT). The mean rank (MR) of all parameters, their standard deviation (SDR), as well as the grand mean rank (GMR) were determined. Estimates of genetic parameters were done according to the procedures cited in Ajayi et al., (2017a). The data on the ranking of accessions were subjected to cluster analysis using the Paleontological statistics software package for data analysis (PAST) version 4.0 (Hammer et al., 2001). All final data for wilting, morphological, physiological, recovery and yield data were used for Principal Component Analysis (PCA) employing PAST.

3. Results

3.1. Variability among accessions of cowpea under drought stress at the flowering stage

Results from ANOVA revealed highly significant differences among accessions for all traits. The coefficient of variation among traits ranged between 4.71 in leaf wilting index (LWI) on day 21 and 42.17% in the percentage of wilted plants (PPW) on day 14 (Table 3). Wilting parameters of the accessions are presented in Table 4. The drought susceptibility score (DSS) and PPW, respectively ranged between 2.61 and 26.33% in AC06 to 6.84 and 97.33% in AC03 as of day 21 of drought stress. However, LWI ranged between 0.81 in AC06 and 1.00 in AC01, AC03, and AC09.

Table 3. Mean square values for all parameters of accessions of cowpea screened under drought stress at the flowering stage

Parameters	Accession (DF = 9)	Error (DF = 20)	CV (%)
DSS14	4.37**	0.60	27.66
DSS21	7.59**	0.99	20.23
PPW14 (%)	1186.82**	76.70	42.17
PPW21 (%)	2080.62**	262.25	24.30
LWI7	0.05**	0.01	40.00
LWI14	0.04**	0.03	21.92
LWI21	0.01**	0.002	4.71
IRWC (%)	29.32**	23.42	10.19
RWC7 (%)	163.33**	73.96	12.06
RWC14 (%)	235.38**	86.45	17.47
SCND (mmol $m^{-2}s^{-1}$)	685.34**	106.06	11.55
PREC (%)	1812.87**	29.50	23.54
STG	17.28**	0.24	17.69
STR	4.61**	0.26	36.68
ITLL (cm)	2.29**	1.34	9.86
TLL7 (cm)	2.21**	1.37	10.16
ITLW (cm)	5.09**	0.76	11.89
TLW7 (cm)	5.27**	0.74	12.01
ISG (mm)	0.26**	0.06	11.25
SG7 (mm)	0.28**	0.24	10.45
SG14 (mm)	0.94**	0.28	13.96
PPSET (%)	534.81**	56.67	19.47
TPDP	7.11**	1.00	25.38
SPP	13.48**	1.35	10.08
SDPL	1034.66**	188.89	30.86
PDL (cm)	8.68**	0.82	5.88
SYDPL (g)	19.82**	2.33	23.52
PODW (g)	0.05**	0.008	24.17

**: Significant at $P \leq 0.05$; DF: Degree of freedom

DSS14, 21: Drought susceptibility score at day 14 and 21; PPW14, 21: Percentage of plants wilted at day 14 and 21; LWI7, 14, 21: Leaf wilting index at day 7, 14, 21; IRWC, RWC7, RWC14: Initial relative water content, relative water content at day 7, relative water content at day 14; SCND: Stomata conductance; PREC: Percentage recovery; STG: Stem greenness; STR: Stem re-growth; ITLL: Initial terminal leaflet length; TLL7: Leaflet length at day 7; ITLW: Initial terminal leaflet width; TLW7: Leaflet width at day 7; ISG: Initial stem girth; SG7, 14, 21: Stem girth at day 7, 14 and 21; PPSET: Percentage pod set; TPDP: Total pods per plant; SPP: Seeds per pod; SDPL: Seeds per plant; PDL: Pod length; SYDPL: Seed yield per plant; PODW: Pod weight

Table 4. Wilting parameters (means ± standard error) of accessions of cowpea screened under drought stress at the flowering stage

ACCESSION	DSS 14	DSS 21	PPW 14	PPW 21	LWI 7	LWI 14	LWI 21
AC01	2.68±0.39^{ab}	6.16±0.47^{c}	13.33±3.33^{ab}	97.00±1.73^{e}	0.24±0.03^{abc}	0.93±0.06^{a}	1.00±0.00^{c}
AC02	2.54±0.69^{ab}	5.02±0.77^{bc}	20.00±5.77^{bc}	64.00±15.50^{cd}	0.37±0.07^{cd}	0.83±0.04^{a}	0.92±0.04^{bc}
AC03	3.57±0.77^{bc}	6.84±0.11^{c}	50.67±10.53^{ef}	97.33±2.67^{e}	0.52±0.12^{d}	0.87±0.12^{a}	1.00±0.00^{c}
AC04	1.75±0.13^{a}	2.85±0.23^{a}	8.67±0.67^{ab}	28.33±7.26^{ab}	0.14±0.04^{ab}	0.67±0.04^{a}	0.85±0.02^{ab}
AC05	2.07±0.29^{a}	3.60±0.32^{ab}	0.00±0.00^{a}	53.33±3.33^{abc}	0.21±0.04^{abc}	0.82±0.07^{a}	0.98±0.02^{c}
AC06	1.91±0.35^{a}	2.61±0.35^{a}	0.00±0.00^{a}	26.33±4.91^{a}	0.22±0.07^{abc}	0.64±0.12^{a}	0.81±0.05^{a}
AC07	1.97±0.29^{a}	5.05±0.60^{bc}	28.00±4.36^{cd}	65.33±6.89^{cd}	0.28±0.05^{bc}	0.67±0.09^{a}	0.94±0.00^{bc}
AC08	5.37±0.55^{d}	6.77±0.12^{c}	60.00±5.77^{f}	90.27±5.00^{bc}	0.25±0.06^{abc}	0.94±0.03^{a}	0.97±0.02^{c}
AC09	1.96±0.17^{a}	4.00±1.16^{ab}	0.00±0.00^{a}	56.67±17.64^{bc}	0.04±0.01^{a}	0.69±0.17^{a}	1.00±0.00^{c}
AC10	4.21±0.41^{cd}	6.29±0.65^{c}	37.00±3.79^{de}	87.66±12.33^{de}	0.23±0.06^{abc}	0.92±0.07^{a}	0.99±0.01^{c}

Means with the same alphabet within a column are not significantly different from one another at $P \leq 0.05$ using DMR.
DSS14, 21: Drought susceptibility score at day 14 and day 21; PPW14, 21: Percentage of wilted plants at day 14 and 21; LWI7, 14, 21: Leaf wilting index at day 7, 14, and 21.

The morphological parameters of the accessions, such as terminal leaf length (TLL), terminal leaf width (TLW), and stem girth are presented in Table 5. The final TLL measured on day 7 of stress was highest (13.07 cm) in AC08 and lowest (10.03 cm) in AC07. However, the percentage reduction between initial and final ranged between the lowest (-0.36%) in AC05 and the highest (8.24%) in AC02. Also, TLW ranged between 5.30 cm in AC07 and 9.30 cm in AC04, with the lowest percentage reduction (-0.35%) in width in AC05 and the highest reduction (8.77%) in AC01. Further, the highest reduction (33.17%) of stem girth as of day 21 of stress occurred in AC01 with the least stem girth of 2.80 mm, meanwhile, both the lowest percentage reduction of stem girth (6.51%) and biggest stem girth (4.74 mm) respectively were obtained in AC09.

The yield traits of the accessions under drought stress are presented in Table 6. The highest performance for seed yield pod length (18.30 cm), (9.95 g), and pod weight (0.56 g) was obtained in AC01 while the lowest performance for seed yield (2.75 g), pod length (12.27 cm), and pod weight, respectively was obtained in AC10 and AC09. Performance for the number of seeds per pod (1.27) and the number of seeds per plant (82.37) was highest in AC03 while the lowest performance in the two traits respectively was obtained in AC07 (8.93) and AC04 (19.66). However, the percentage of pod set per plant ranged from the lowest (16.67%) in AC06 to the highest (56.66%) in AC05 while the total number of pods per plant ranged from the lowest (1.73) in AC04 to the highest (6.48) in AC07.

The results for physiological parameters such as relative water content (RWC) and stomatal conductance (SCND) are presented in Table 7. The final RWC observed on day 14 of drought stress was highest (63.79%) in AC07 and lowest (41.01%) in AC10, consequently, the highest reduction in the trait (47.84%) between the initial and final value was obtained in AC03 while the lowest reduction (14.05%) was obtained in AC09. SCND on the hand was observed to range from the lowest (72.58 mmol $m^{-2}s^{-1}$) in AC09 and (119.38mmol $m^{-2}s^{-1}$) in AC10.

Table 5. Effect of drought stress on terminal leaflet length and width (cm), and stem girth (mm) (means ± standard error) of accessions of cowpea at the flowering stage

ACCESSION	ITLL	TLL7	RD (%)	ITLW	TLW7	RD (%)	ISG	SG7	SG14	RD (%)
AC01	11.50±0.75^{ab}	11.47±0.58^{ab}	0.26	6.50±0.46^{abc}	5.93±0.49^{ab}	8.77	4.19±0.25^{a}	4.15±0.21^{a}	2.80±0.43^{a}	33.17
AC02	12.13±0.75^{ab}	11.13±1.17^{ab}	8.24	6.60±0.70^{abc}	6.53±0.69^{ab}	1.06	4.31±0.05^{ab}	4.41±0.37^{ab}	3.31±0.41^{ab}	23.2
AC03	11.70±0.72^{ab}	11.30±0.55^{ab}	3.42	7.90±0.55^{cde}	7.57±0.43^{bc}	4.18	4.87±0.24^{cd}	4.62±0.23^{ab}	3.71±0.14^{abc}	23.81
AC04	12.63±0.86^{b}	12.57±0.83^{b}	0.48	9.37±0.47^{e}	9.30±0.46^{d}	7.47	4.71±0.19^{bcd}	4.69±0.28^{ab}	3.89±0.55^{bcd}	17.41
AC05	11.23±0.62^{ab}	11.27±0.64^{ab}	-0.36	8.60±0.70^{de}	8.63±0.77^{cd}	-0.35	4.59±0.04^{abc}	4.66±0.15^{ab}	3.70±0.14^{abc}	19.39
AC06	11.93±0.63^{ab}	11.80±0.66^{ab}	1.08	8.70±0.46^{de}	8.50±0.47^{cd}	2.29	5.09±0.08^{d}	5.22±0.19^{b}	4.60±0.09^{cd}	9.63
AC07	10.03±0.23^{a}	10.03±0.23^{a}	0.00	5.30±0.32^{a}	5.30±0.32^{a}	0.00	4.67±0.04^{bcd}	4.67±0.18^{ab}	3.83±0.03^{bcd}	17.99
AC08	13.27±1.01^{b}	13.07±0.86^{b}	1.51	6.10±0.46^{ab}	5.97±0.49^{ab}	2.13	4.81±0.14^{cd}	4.60±0.26^{ab}	3.59±0.34^{abc}	25.36
AC09	11.83±0.35^{ab}	11.80±0.36^{ab}	0.25	6.70±0.15^{abc}	6.60±0.06^{ab}	1.49	5.04±0.04^{cd}	5.09±0.21^{ab}	4.74±0.19^{d}	6.51
AC10	11.17±0.46^{ab}	10.80±0.32^{ab}	3.31	7.53±0.52^{bcd}	7.23±0.40^{bc}	3.98	4.94±0.08^{cd}	4.83±0.52^{ab}	3.77±0.29^{abcd}	23.68

Means with the same alphabet within a column are not significantly different from one another at $P \leq 0.05$ DMRT. RD: Percentage reduction. ITLL: Initial terminal leaflet length; TLL 7: Terminal leaflet length at day 7; ITLW: Initial terminal leaflet width; TLW 7: Terminal leaflet width at day 7; ISG: Initial stem girth; SG7, 14: Stem girth at day 7, and 14.

Table 6. Yield traits (means ± standard error) of accessions of cowpea screened under drought stress at the flowering stage

ACCESSION	PPSET (%)	TPDP	SPP	SDPL	PDL (cm)	SYDPL (g)	PODW (g)
AC01	53.33±3.33^{ef}	4.73±0.24^{cde}	11.13±0.07^{bcd}	52.72±2.90^{bc}	18.30±0.88^{e}	9.95±0.36^{d}	0.56±0.02^{e}
AC02	43.33±6.67^{def}	4.87±0.47^{cde}	11.47±0.35^{bcd}	55.87±6.08^{c}	15.36±0.24^{bcd}	9.49±1.03^{cd}	0.42±0.05^{cde}
AC03	50.00±5.77^{ef}	5.35±1.28^{de}	15.27±0.47^{f}	82.37±21.63^{d}	16.63±0.16^{d}	6.82±1.90b^{bc}	0.27±0.01^{abc}
AC04	26.66±3.33^{abc}	1.73±0.27^{a}	11.78±1.64^{cd}	19.66±2.07^{a}	14.44±0.15^{bc}	2.78±0.22^{a}	0.53±0.12^{de}
AC05	56.66±3.33^{f}	3.27±0.51^{abc}	13.10±0.45^{de}	42.55±6.19^{abc}	16.87±0.41^{de}	5.98±0.95^{c}	0.40±0.04^{bcde}
AC06	16.67±3.33^{a}	1.89±0.11^{a}	14.29±0.46^{ef}	27.04±2.03^{ab}	15.78±0.11^{cd}	4.55±0.32^{ab}	0.38±0.07^{bcd}
AC07	43.33±3.33^{def}	6.48±0.52^{e}	8.93±0.24^{a}	57.79±4.11^{c}	13.76±0.29^{ab}	8.99±0.83^{cd}	0.29±0.03^{abc}
AC08	23.33±3.33^{ab}	4.43±0.54^{bcd}	9.93±0.44^{abc}	43.71±4.07^{abc}	15.68±0.65^{cd}	6.65±0.58^{bc}	0.44±0.06^{cde}
AC09	40.00±5.77^{cde}	4.00±0.58^{bcd}	9.33±0.88^{ab}	36.67±4.17^{abc}	12.27±0.91^{a}	6.98±0.80^{bc}	0.17±0.03^{a}
AC10	33.33±3.33^{bcd}	2.67±0.44^{ab}	10.10±0.10^{abc}	26.91±4.37^{ab}	14.85±0.57^{bc}	2.75±0.48^{a}	0.25±0.02^{ab}

Means with the same alphabet within a column are not significantly different from one another at $P \leq 0.05$ using DMRT. PPSET: Percentage pod set; TPDP: Total pods per plant; SPP: Seeds per pod; SDPL: Seeds per plant; PDL: Pod length; SYDPL: Seed yield per plant; PODW: Pod weight.

Table 7. Effect of drought stress on the physiological parameters (means ± standard error) of accessions of cowpea at the flowering stage

ACCESSION	IRWC (%)	RWC 7 (%)	RWC 14 (%)	RD (%)	SCND (mmol $m^{-2}s^{-1}$)
AC01	86.47±0.48^{ab}	80.51±6.71^{bc}	47.59±9.58^{abc}	44.96	90.43±3.45^{a}
AC02	89.50±0.78^{b}	77.04±7.19^{bc}	59.98±8.82^{bcd}	32.98	85.52±1.81^{a}
AC03	84.66±0.70^{ab}	59.09±8.94^{a}	44.16±0.59^{ab}	47.84	86.79±7.07^{a}
AC04	80.46±1.82^{ab}	75.56±5.17^{abc}	59.72±7.07^{bcd}	25.78	77.08±4.69^{a}
AC05	83.88±1.48^{ab}	69.96±0.48^{abc}	51.56±2.55^{abcd}	38.53	86.21±2.09^{a}
AC06	85.24±2.09^{ab}	70.88±4.41^{abc}	50.56±3.95^{abcd}	40.69	82.83±3.42^{a}
AC07	83.16±2.17^{ab}	66.66±1.89^{abc}	63.79±2.81^{cd}	23.29	78.72±8.60^{a}
AC08	84.52±2.44^{ab}	64.72±3.12^{ab}	46.72±2.19^{abc}	44.72	112.48±12.49^{b}
AC09	78.02±0.73^{a}	82.06±1.44^{c}	67.06±0.74^{d}	14.05	72.58±2.09^{a}
AC10	84.55±7.45^{ab}	66.61±2.84^{abc}	41.01±5.72^{a}	51.49	119.38±3.99^{b}

Means with the same alphabet within a column are not significantly different from one another at $P \leq 0.05$ using DMRT. RD: Percentage reduction. IRWC: Initial relative water content; RWC 7, 14: Relative water content at days 7 and 14; SCND: Stomata conductance.

The recovery parameters such as percentage of recovered plants (PREC), stem re-growth (STR), and greenness (STG) are presented in Table 8. Accessions AC01, AC03, AC08, and AC10 did not recover. AC06, on the other hand, had the highest PREC (65.00%), the highest STR (3.11), and the highest STG (4.89).

Accession ranks based on wilting parameters, reduction in morphological traits, RWC, stomatal conductance, and yield traits are presented in Table 9. The mean of ranks (MR) and standard deviation of ranks (SDR) respectively, were highest in AC10 (7.39) and AC09 (3.18), while the lowest values were obtained in AC05 (4.00) and AC02 (1.85). Based on the MR and SDR, accessions with a mean rank lower than the grand mean (GM) of ranks (5.32) and its SDR (2.59) were classified as the highly tolerant accessions; these included AC02 and AC05. Moderately tolerant accessions included those with lower MR values but higher SDR compared to GM, such as AC06, AC07, and AC09. Moderately susceptible accessions were those with MR a bit higher than the GM and with higher SDR, including AC01 and AC04. Highly susceptible accessions were AC03, AC08, and AC10 with higher MR (>5.99) compared to GM.

Table 8. Recovery parameters (means ± standard error) of accessions of cowpea screened under drought stress at the flowering stage after two weeks of re-watering

ACCESSION	PREC (%)	STG	STR
AC01	0.00±0.00^{a}	1.00±0.00^{a}	0.00±0.00^{a}
AC02	20.00±3.00^{b}	4.72±0.15^{bc}	1.83±0.44^{b}
AC03	0.00±0.00^{a}	1.00±0.00^{a}	0.00±0.00^{a}
AC04	43.33±5.77^{c}	4.68±0.09^{bc}	2.07±0.52^{b}
AC05	21.66±7.64^{b}	4.67±0.17^{bc}	2.33±0.33^{bc}
AC06	65.00±8.66^{d}	4.89±0.11^{c}	3.11±0.11^{c}
AC07	24.00±6.08^{b}	3.92±0.85^{b}	2.22±0.39^{bc}
AC08	0.00±0.00^{a}	1.00±0.00^{a}	0.00±0.00^{a}
AC09	56.66±9.07^{d}	4.83±0.17^{bc}	2.33±0.33^{bc}
AC10	0.00±0.00^{a}	1.00±0.00^{a}	0.00±0.00^{a}

Means with the same alphabet within a column are not significantly different from one another at $P \leq 0.05$ using DMRT. PREC: Percentage recovery; STG: Stem greenness; STR: Stem re-growth.

Table 9. Ranking of accessions of cowpea screened under drought stress at flowering stage

ACC	DSS21	PPW21 (%)	LWI21	RRWC (%)	PREC (%)	STG	STR	SCND (mmol $m^{-2}s^{-1}$)	RTLL (%)	RTLW (%)	RSG (%)	PPSET (%)	TPDP	SPP	SDPL	PDL	SYDPL (g)	PODW (g)	MR	SDR
AC01	7	9	8	8	7	7	6	3	4	10	10	2	4	6	4	1	1	1	5.44	3.05
AC02	5	5	3	4	6	3	5	6	10	3	6	4	3	5	3	6	2	4	4.61	1.85
AC03	10	10	8	9	7	7	6	4	9	8	8	3	2	1	1	3	5	8	6.06	3.04
AC04	2	2	2	3	3	4	4	9	5	9	3	7	10	4	10	8	9	2	5.33	3.07
AC05	3	3	6	5	5	5	2	5	1	1	5	1	7	3	6	2	7	5	4.00	2.03
AC06	1	1	1	6	1	1	1	7	6	6	2	9	9	2	8	4	8	6	4.39	3.13
AC07	6	6	4	2	4	6	3	8	2	2	4	4	1	10	2	9	3	7	4.61	2.64
AC08	9	8	5	7	7	7	6	2	7	5	9	8	5	8	5	5	6	3	6.22	1.93
AC09	4	4	8	1	2	2	2	10	3	4	1	5	6	9	7	10	4	10	5.11	3.18
AC10	8	7	7	10	7	7	6	1	8	7	9	6	8	7	9	7	10	9	7.39	2.00
GM																			**5.32**	**2.592**

ACC: Accessions; MR: Mean of rank; SDR: Standard deviation of rank; GM: Grand mean of rank.
DSS21: Drought susceptibility score at day 21; PPW21: Percentage of plants wilted at day 21; LWI21: Leaf wilting index at day 21; RRWC: Percent reduction in relative water content by day 14; SCND: Stomata conductance; PREC: Percentage recovery; STG: Stem greenness; STR: Stem re-growth; RTLL: Percent reduction in leaflet length by day 7; RTLW7: Percent reduction in leaflet width by day 7; Percent reduction in stem girth by day 14; PPSET: Percentage pod set; TPDP: Total pods per plant; SPP: Seeds per pod; SDPL: Seeds per plant; PDL: Pod length; SYDPL: Seed yield per plant; PODW: Pod weight.

3.2. Cluster analysis based on the ranking of accessions of cowpea screened under drought stress at the flowering stage

The ranking of accessions under drought stress produced a Dendrogram that grouped the accessions into three major clusters (Figure 1). Cluster I consisted of AC10, AC08, AC01, and AC03, both highly susceptible and moderately susceptible accessions. Cluster II had three sub-clusters; sub-cluster II-a consisted of AC07 and AC09, moderately tolerant accessions. Sub-cluster II-b and c respectively, consisted of AC02 and AC05, highly tolerant accessions. However, cluster III consisted of AC06 (moderately tolerant accession) and AC04 (a moderately susceptible accession).

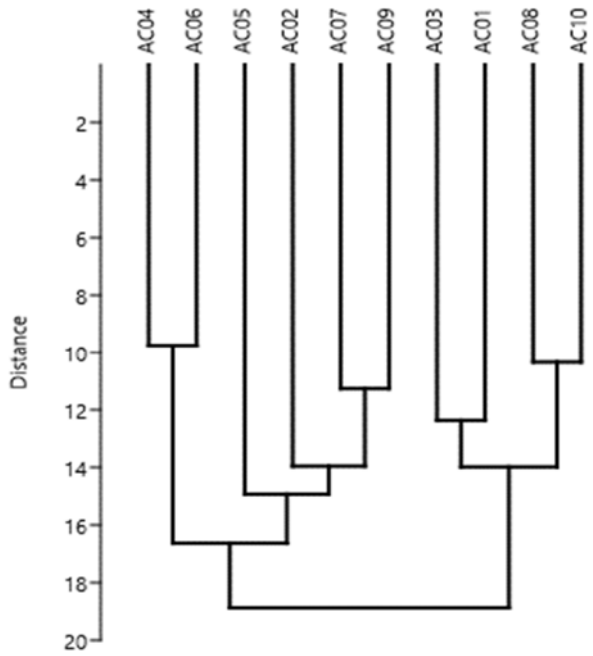

Figure 1. Dendrogram (UPGMA) based on the ranking of accessions of cowpea screened under drought stress at the flowering stage

3.3. Estimates of genetic parameters of accessions of cowpea screened under drought stress at the flowering stage

The results for estimates of genetic parameters are presented in Table 10. Genotypic variance (GV) ranged from 0.003 in LWI14 and 21 to 606.12 in PPW21, while the phenotypic variance (PV) fell between 0.005 in LWI21 and 868.37 in PPW21. Genotypic coefficient of variation (GCV = 105.69%) and phenotypic coefficient of variation (PCV = 108.28%) respectively were highest in PREC. However, the lowest GCV (1.67%) and PCV (5.99%) were obtained in the IRWC. Heritability in the broad sense (H^2b) ranged from 7.76% in IRWC to the highest (95.95) in STG. Furthermore, genetic advance as a percent of the mean (GAM) ranged from the lowest (0.01%) in IRWC to the highest (173.64%) in PPW14. Heritability was high in all wilting traits except in LWI7 and 14. It was high for all recovery parameters and yield traits except for the number of seeds per plant and low for all morphological and physiological parameters except for TLW and stomatal conductance.

3.4. PCA and biplot based on all final parameters of accessions of cowpea screened under drought stress at the flowering stage

The PCA and biplot respectively are presented in Table 11 and Figure 2. Nine PC axes were derived, with the first four having eigenvalues above 1.00 and accounting for 88.26% of the total variation. PCs 1 and 2 respectively, accounted for 44.70% and 21.36% of the variability, totaling 66.06%. All parameters had high contributions in PC1 except TLL7, seeds per pod, and pod weight. However, all the wilting parameters, SG14 and PREC the ones with low contributions in PC2. Biplot based on PCs 1 and 2 divided accessions into four major groups. Group, I consisted of two, AC07 (moderately tolerant) and AC02 (highly tolerant). Group II consisted of four, AC01 (moderately susceptible), AC03, AC08, and AC10 (highly susceptible). Group III consisted of three, AC06 (moderately tolerant), AC04 (moderately susceptible), and AC05 (highly tolerant). Group IV consisted of one, AC09 (moderately tolerant). All the yield parameters except pod length and weight were highly positively correlated with LWI21 in group I and weakly correlated with DSS21 and PPW21 in group II. However, pod length and stomatal conductance were highly positively correlated with each other and also weakly positively

correlated with DSS21 and PPW21 in group II. In group III, TLW7 and TLL7 were highly positively correlated and also weakly correlated with pod length and pod weight, and highly negatively correlated with all other yield parameters. All recovery parameters in group IV were highly positively correlated and correlated positively with SG14 and RWC14, and highly negatively correlated with all the yield and wilting parameters. Vertex accession for yield traits in group I was AC07, while the vertex accessions in group II for the wilting parameters and SCND were AC10, AC08, and AC01. The vertex accessions in group III were AC06 and AC04 for TLW7 and TLL7, while the vertex accession for recovery and RWC14 was AC09. The most stable accessions under drought stress were AC05 and AC02 because of their very close positions to the origin of the biplot.

Table 10. Estimates of genetic parameters of accessions of cowpea screened under drought stress at flowering stage

TRAIT	MEAN	GV	PV	GCV (%)	PCV (%)	H^2B (%)	GAM (%)
DSS14	2.80	1.26	1.86	40.09	48.71	67.74	67.97
DSS21	4.92	2.20	3.19	30.15	36.30	68.97	51.58
PPW14 (%)	20.77	370.04	446.74	92.61	101.76	82.83	173.64
PPW21 (%)	66.63	606.12	868.37	36.95	44.23	69.79	63.58
LWI7	0.25	0.01	0.02	40.00	56.57	50.00	58.27
LWI14	0.79	0.003	0.03	6.93	21.92	31.61	14.28
LWI21	0.95	0.003	0.005	5.77	7.44	60.00	9.19
IRWC (%)	84.05	1.97	25.39	1.67	5.99	7.76	0.01
RWC7 (%)	71.31	29.79	103.75	7.65	14.28	28.71	8.45
RWC14 (%)	53.22	49.64	136.09	13.26	21.97	36.48	16.47
SCND	89.20	193.00	299.06	15.57	19.39	64.54	25.77
PREC (%)	23.07	594.46	623.96	105.69	108.28	95.27	212.61
STG	2.77	5.68	5.92	86.04	87.84	95.95	173.61
STR	1.39	1.45	1.71	86.63	94.08	84.79	164.32
ITLL (cm)	11.74	0.32	1.66	4.82	10.97	19.28	4.36
TLL7 (cm)	11.52	0.28	1.65	4.59	11.15	16.97	3.89
ITLW (cm)	7.33	1.44	2.20	16.37	20.24	65.45	27.28
TLW7 (cm)	7.16	1.51	2.25	17.16	20.95	67.11	28.96
ISG (mm)	4.74	0.07	0.13	11.83	16.57	53.85	8.44
SG7 (mm)	4.69	0.01	0.25	2.13	10.66	4.00	0.87
SG14 (mm)	3.79	0.22	0.50	12.38	18.66	44.00	16.91
PPSET (%)	38.67	159.38	216.05	32.65	38.01	73.77	57.76
TPDP	3.94	2.04	3.04	36.25	44.25	67.11	61.17
SPP	11.53	4.04	5.39	17.43	20.14	74.95	31.09
SDPL	44.53	281.92	470.81	37.71	48.73	59.88	60.11
PDL (cm)	15.39	2.62	3.44	10.52	12.05	76.16	18.91
SDYPL (g)	6.49	5.83	8.16	37.2	44.01	71.45	64.78
PODW (g)	0.37	0.014	0.022	31.98	40.09	63.63	52.55

GV: Genotypic variance; PV: Phenotypic variance; GCV: Genotypic coefficient of variation; PCV: Phenotypic coefficient of variation; H^2B: Heritability; GAM: Genetic advance as percent of the mean. DSS14, 21: Drought susceptibility score at days 14 and 21; PPW14, 21: Percentage of plants wilted at day 14 and 21; LWI7, 14, 21: Leaf wilting index at day 7, 14, and 21; IRWC: Initial relative water content; RWC 7, 14: Relative water content at day 7 and 14; SCND: Stomata conductance; PREC: Percentage recovery; STG: Stem greenness; STR: Stem re-growth; ITLL: Initial terminal leaflet length; TLL7: Terminal leaflet length at day 7; TLW7: ITLW: Initial terminal leaflet width; Terminal leaflet width at day 7; ISG: Initial stem girth; SG, 7, 14: Stem girth at day 7, and 14; PPSET: Percentage pod set; TPDP: Total pods per plant; SPP: Seeds per pod; SDPL: Seeds per plant; PDL: Pod length; SDYPL: Seed yield per plant; PODW: Pod weight.

Table 11. PCA of accessions of cowpea screened under drought stress at flowering stage

Parameters	PC1	PC2	PC3	PC4
DSS21	**0.95**	-0.05	-0.24	0.02
PPW21 (%)	**0.98**	-0.01	-0.16	0.06
LWI21	**0.77**	0.24	-0.22	0.22
RWC14 (%)	**-0.54**	**0.75**	-0.03	-0.29
SCND (mmol $m^{-2}s^{-1}$)	**0.55**	**-0.60**	**-0.45**	-0.05
TLL7 (cm)	-0.22	**-0.55**	-0.07	**-0.40**
TLW7 (cm)	**-0.62**	**-0.55**	0.32	0.36
SG14	**-0.74**	0.12	**-0.43**	0.39
PREC (%)	**-0.94**	0.21	-0.01	0.03
STG	**-0.85**	**0.41**	0.21	-0.05
STR	**-0.88**	0.37	0.19	0.02
PPSET (%)	**0.52**	**0.46**	**0.46**	0.23
TPDP	**0.65**	**0.70**	0.06	-0.05
SPP	-0.12	**-0.40**	**0.66**	**0.55**
SDPL	**0.67**	**0.43**	0.36	0.28
PDL (cm)	**0.50**	**-0.46**	**0.68**	-0.01
SYDPL (g)	**0.49**	**0.68**	0.35	-0.30
PODW (g)	0.03	**-0.42**	**0.59**	**-0.68**
Eigen-values	8.05	3.85	2.42	1.58
Variance (%)	44.70	21.36	13.42	8.78
Cum. Variance (%)	44.70	66.06	79.48	88.26

High contribution (≥4.0).

DSS21: Drought susceptibility score at day 21; PPW21: Percentage of plants wilted at 21; LWI21: Leaf wilting index at day 21; RWC14: Relative water content at day 14; SCND: Stomatal conductance; PREC: Percentage recovery; STG: Stem greenness; STR: Stem re-growth; TLL7: Terminal leaflet length at day 7; TLW7: Terminal leaflet width at day 7; SG14: Stem girth at day 14; PPSET: Percentage pod set; TPDP: Total pods per plant; SPP: Seeds per pod; SDPL: Seeds per plant; PDL: Pod length; SYDPL: Seed yield per plant; PODW: Pod weight.

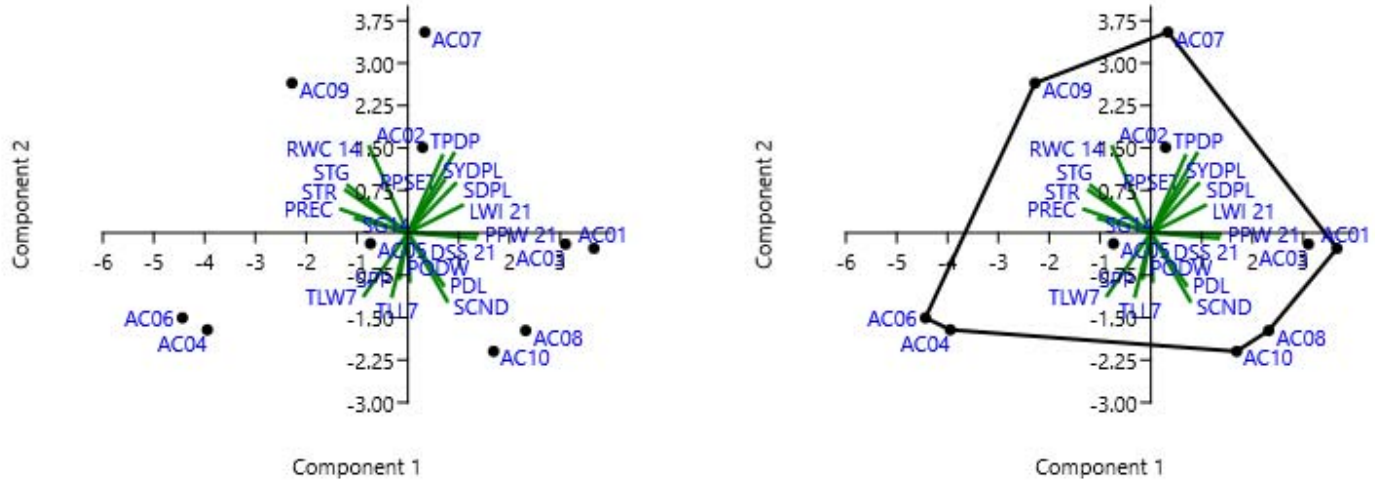

Figure 2. Biplot of PCA (a) and polygon view of biplot (b) of accessions of cowpea screened under drought stress at flowering stage. ACC01 – ACC10 are codes for accessions. DSS21: Drought susceptibility score at day 21; PPW21: Percentage of plants wilted at 21; LWI21: Leaf wilting index at day 21; RWC14: Relative water content at day 14; SCND: Stomatal conductance; PREC: Percentage recovery; STG: Stem greenness; STR: Stem re-growth; TLL7: Terminal leaflet length at day 7; TLW7: Terminal leaflet width at day 7; SG14: Stem girth at day 14; PPSET: Percentage pod set; TPDP: Total pods per plant; SPP: Seeds per pod; SDPL: Seeds per plant; PDL: Pod length; SYDPL: Seed yield per plant; PODW: Pod weight.

4. Discussion

4.1. Accession differences for measured parameters under drought stress at the flowering stage

The high significant variability revealed by the ANOVA among accessions for all traits studied at the flowering stage indicated the existence of sufficient genetic variations among the accessions. This agrees with Nkouannesssi (2005). Visual observation of wilting resulting from drought stress is possible in cowpea, and this allows the classification of genotypes based on the intensity of wilting. It was observed from the results that responses were accession-specific and time-dependent. Wilting parameters showed that as early as day 7, accessions AC02 and AC03 were already showing signs of wilting as conveyed in Nkouannessi (2005), Muchero et al., (2008), Agbicodo (2009), Nkomo et al. (2020), suggesting that susceptible accessions can easily be identified within the first week of withholding moisture (Pungulani et al., 2013). However, the most susceptible on day 21 were AC03, AC08, AC01, and AC10 with a combination of the highest PPW and LWI. This is similar to the responses of the accessions at the seedling and vegetative stages (Ajayi et al., 2018; 2020).

Re-watering of accessions for two weeks after the stress resulted in the recovery of some plants among six accessions excluding AC01, AC03, AC08, and AC10 in which all plants have died and dried. Among accessions, those in which wilting was slow at the beginning with consistently lower DSS and lower PPW at the end such as AC04, AC06, and AC09 had very high recovery rates. Therefore, these accessions must have had an in-built mechanism to retard their tissue moisture loss by restricting the rate of transpiration that the highly susceptible accessions did not possess. This backed their reduced predisposition to drought stress and their possession of higher greenness at recovery. The importance of stem greenness to drought tolerance in cowpea has been previously emphasized (Guofo et al., 2017). Similar results were recorded during the seedling and the vegetative stages (Ajayi et al., 2018; 2020) and also in line with other reports on soybean, common bean, and cowpea (Ries et al., 2012; Fatokun et al., 2012; Mukeshimana et al., 2014; Ibitoye, 2015; Nkomo et al., 2020).

Pungulani et al. (2013) stated that the physiological and morphological traits promoting water loss from leaf tissues might be a key factor responsible for early wilting among the accessions showing droopiness within seven days of being stressed. In sorghum, drought stress was

reported to negatively affect most morphological and physiological traits (Bibi et al., 2010; 2012). Stomata behavior at the initiation of drought stress might be a crucial factor since late wilting accessions were able to regulate stomatal opening to prevent water loss (Agbicodo, 2009).

Generally, the accessions had high stomatal conductance. Plants of the accessions with the slowest rate of wilting (AC04, AC05, AC06, AC07, and AC09) were among those with the lowest stomatal conductance. These results show that these accessions can maintain photosynthesis under restricted moisture through minimal stomatal openings as reported by Mukeshimana (2013). Other mechanisms of restricting moisture loss during drought stress also include an osmotic adjustment in leaves (White et al., 1992) which encourages the maintenance of turgor and survival in plants under drought stress and ensures plant recovery after re-introduction of watering (Mukeshimana, 2013).

In the present study, RWC was reduced among accessions at the flowering stage up to day 14 of drought stress. Furthermore, an RWC of above 40% among cowpea genotypes is considered one of the best indicators of drought tolerance (Alidu *et al*., 2019). In the present study, the tolerant accessions possessed an RWC of between 50 and 67% in line with Alidu et al. (2019) for inbred lines of cowpea. Accessions with slow wilting had the highest RWC on day 14 of drought stress. Reduction of RWC between the initial and day 14 was generally the lowest among accessions with low wilting parameters. Maintenance of higher water status among tolerant accessions could be linked to lower stomatal conductance exhibited by them in line with the findings of Shanmugam et al. (2021) in rice. This result contradicts the findings of Agbicodo (2009) who observed no correlation between the leaf water status of cowpea plants and stomatal conductance under water deficit. Inconsistent reports have emanated from many workers as regards relationships between RWC and stomatal conductance in cowpea plants under drought stress (Anyia and Herzog, 2004; Souza *et al*., 2004; Hamidou *et al*., 2007). RWC is a vital agronomic parameter that governs better growth and physiological functions in plants. Plants growing under drought conditions can experience increased osmotic potential leading to a decrease of 60 – 80% in RWC, which can be most pronounced among the highly sensitive accessions (Panda et al., 2022) in agreement with the findings of this study. For instance, the most sensitive accessions; AC10, AC03, and AC08, were among those in which RWC reduced the most as a result of drought.

Observable differences were noted among the accessions for morphological traits such as TLL and TLW under drought stress until day 7. The significant reduction in the TLL and TLW among the most susceptible accessions at day 7 indicates that drought stress led to

reduced leaf area of existing leaves which in turn led to leaf shedding on the plants because many of the measured terminal leaflets did not survive beyond day 7. The highly sensitive accessions were amongst those with the highest reduction in TLL and TLW (AC03, AC08, and AC10). These are similar to the findings of Samson and Helmut (2007) and also consistent with their responses at the seedling and vegetative stages (Ajayi et al., 2018; 2020). Also, the most susceptible at the flowering stage were among accessions with the highest reduction of stem girth resulting from drought stress. These findings are in agreement with the findings of Abdou Razakou (2013) who conveyed that drought imposed on cowpea plants reduced their stem girth between 2.43% and 50%. As stated by Mitchell et al., (1998) and Abdou Razakou (2013), reduction of plant size and leaf area has been found as mechanisms controlling water use and reducing injury under drought stress. Genotypes of maize also differed significantly for stem diameter at different drought stages (Salami et al., 2007).

Severe drought has been found to cause a significant reduction in seed yield which is linked to a reduction in plant water status in many legumes (Garg et al., 2005). Significant genotypic differences were observed among the accessions for the number of pods, percentage of pod set, the total number of pods, seeds per pod, seeds per plant, pod length, seed yield, and pod weight at the flowering stage. The percentage pod set and the number of pods ranged from low to moderate among highly susceptible accessions. Accession with the highest reduction of RWC (AC10) produced the lowest seed yield under imposed drought, while accessions exhibiting a lesser decrease in RWC set higher pod percentage, formed a higher number of seeds, and higher seed yield. Comparable outcomes were conveyed by Kumar et al., (2008). Agbicodo (2009) reported a significant reduction in grain, fodder, and total yields of cowpea, and stated that imposed drought at the early stages of flowering and pod formation enforced significant damage to plant functions and consequently total biomass yield. Similarly, a decrease in yield under drought stress was reported in cowpea (Aniya and Herzog, 2004a and b; Hamidou et al., 2007). In this study, AC04 and AC06 which displayed the least levels of drought susceptibility at the flowering stage had a low percent pod set, the lowest number of pods, and were among accessions with the lowest seed yield. This indicated that these accessions maintained more tissue moisture by redirecting moisture to tissue rather than exhausting it on pod production. This attribute can be linked to the drought avoidance mechanism of cowpea (Shavrukov et al., 2017), however, one of the major difficulties in classifying cowpeas into different classes based on drought avoidance and escape is the overlap that characterizes the two groups.

4.2. Ranking and cluster analysis based on key traits of accessions of cowpea screened for drought tolerance at the flowering stage

A high level of variations for all traits amongst accessions was responsible for the strength of the accession ranking in clearly separating accessions into different classes of tolerance; this is also indicated in the dendrogram. Determination of the most desirable drought-tolerant genotypes employing mean rank, the standard deviation of ranks, and rank-sum of parameters has been previously reported in crop species (Al-Rawi, 2016; Wasae, 2021). Accordingly, it is common for different traits to rank different accessions as tolerant. Hence, the determination of the most tolerant accessions needs to consider the mean of ranks in conjunction with their standard deviation for all parameters measured. Accessions exhibiting the lowest mean of ranks coupled with the lowest standard deviation of ranks are selected as the most drought–tolerant. The most susceptible accessions of cowpea were separated by the dendrogram from the moderate and the highly tolerant accessions. A similar trend was also observed by Ajayi et al., (2018) for the seedling stage. Cluster analysis is a powerful tool that has been employed in the selection of several drought-tolerant crop species (Zdravkovic et al., 2013; Al-Rawi, 2016).

4.3. PCA and biplot of traits of cowpea screened under drought stress at the flowering stage

According to Hammer et al., (2001), an effective PCA is the one in which most of its variance is accounted for by the first one or first two-component axes. The possession of more than 66 percent of the variance by the first two PCs agrees with Panda et al., (2021) contrary to the findings of (Nkomo et al., 2020). This indicates high variability for drought responses of accessions. All traits except TLL7, seeds per pod, and pod weight were high contributors to PC1. However, only the wilting traits, SG14, PREC, and STR contributed highly to PC2. These traits should be considered in breeding programs for the selection of drought-tolerant cowpea. Li et al., (2015) reported that PCA was effective in selecting the best root parameters governing seedling drought tolerance in maize. The high level of positive correlation shown by the biplot among all the yield parameters, accompanied by the moderate positive correlation with all the wilting parameters indicates that most accessions with high seed yield were more susceptible to drought stress at the flowering stage. Therefore, tolerant accessions (AC07 and AC02) in group I which are highly correlated with the yield traits and LWI21 became sensitive to drought because they produced higher seed yield under drought stress.

High positive correlations among traits such as stomatal conductance and pod length, with their moderate positive correlation with wilting parameters such as DSS21 and PWP21, implies that accessions with high stomatal conductance maintained higher pod length and were more affected by the drought stress. Furthermore, the high negative correlations exhibited between the major yield traits (seed yield, total number of pods per plant, percentage pod set, and seeds per plant) and stomatal conductance suggest that such accessions connected with stomatal conductance in group II (AC08, AC03, AC10, and AC01) were very poor in yield under drought stress. Such accessions can be enhanced through hybridization with the accessions in group I. However, high positive correlations among RWC, recovery parameters, and SG14, imply that ability to maintain high tissue moisture status under drought stress encouraged higher recovery, greenness, regrowth, and higher stem girth of accessions. Therefore, studies on drought tolerance in cowpea should consider wilting parameters with traits such as relative water content, stomatal conductance, yield parameters, and recovery parameters. PCA and biplot align with the findings in both the seedling and vegetative stages (Ajayi et al., 2017b; 2020). Successful selection of drought-tolerant traits and genotypes of sorghum through PCA and biplot have been reported (Bibi et al., 2012).

4.4. Estimates of genetic variation, heritability, and genetic advance of accessions of cowpea screened under drought stress at the flowering stage

It is always helpful to divide phenotypic differences into genetic and non-heritable constituents which include the coefficient of variations on genotypic (GCV), and phenotypic (PCV) levels, heritability, and genetic advance as a percentage of the mean (GAM). These parameters are important for trait improvement and as well as for the effective selection of genotypes for different environmental constraints (Ajayi et al., 2017a). In the present study, GCV was revealed to be lower than PCV for all traits, also, both PCV and GCV were lowered under drought stress compared to the initial values among the wilting parameters except for RWC, TLL, and TLW, similar to the seedling stage (Ajayi et al., 2017a). The reduction of PCV and GCV by drought has been linked to the effect of the environment, suggesting that the performance of genotypes is better under normal growing conditions compared to drought stress (Sabiel et al., 2014). However, GCV and PCV increased under drought stress in the present study in RWC and all morphological traits as reported by Hefny (2013). The marginal differences between GCV and PCV among traits suggest that the traits are mostly governed by the genes. Virtually all traits demonstrated moderate to high heritability under the imposed

drought. Heritability was higher under drought stress in traits such as LWI21, RWC14, and TLW7. Wilting parameters (except LWI), stomatal conductance, TLW7, and all the yield parameters (except seeds per plant) showing both high heritability and GAM under stress suggest that these traits can be used directly to select drought-tolerant cowpea. This is applicable in traits with moderate heritability and high GAM such as seeds per plant, LWI7, LWI14, and RCW14. These findings are similar to the reported data among the same accessions screened under drought stress at the seedling stage. While moderate heritability in RWC agrees with Ajayi (2020), the high heritability for stomatal conductance in the present study is in line with Agbicodo et al., (2009). Heritability was also high for morphological attributes such as the total weight of wheat plants and root weight under drought stress (Naeem et al., 2015). Therefore, centered on heritability and genetic advance, selections for attributes such as wilting traits (DSS, PPW, and LWI), recovery parameters (PREC, STG, and STR), stomatal conductance, and morphological attributes such as stem girth and terminal leaflet width under drought stress would be more effective.

5. Conclusion

Accessions of cowpea varied significantly in their responses to drought stress at the flowering stage. Based on cluster analysis, broad-sense heritability, GAM, PCA, and biplot, selection for traits such as the wilting, RWC, stomatal conductance, terminal leaflet width, and stem girth, and yield parameters such as percentage pod set, the total number of pods, seeds per plant, pod length and seed yield per plant and the recovery parameters under drought stress would be effective. Upon these parameters, four major classes of tolerance were determined: accessions such as AC03, AC08, and AC10 were categorized as highly susceptible. AC01 and AC04 were classified as moderately susceptible. Accessions AC06, AC07, and AC09 were moderately tolerant, while AC02 and AC05 were the highly tolerant accessions. The moderately tolerant and the highly tolerant accessions showed a combination of superior resistance to wilting, superior recovery rates, and superior yield attributes. They also showed lower stomatal conductance, higher RWC, and low reduction of RWC and stem girth under the imposed drought compared to the susceptible ones.

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Research Article

Evaluation of different methods used in morphological examination of canary sperm

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Abstract

By determining the species-specific structure by morphological examination of the sperm, it is possible to improve some of the disadvantages related to short and long-term storage and use in artificial insemination applications. When the morphology of the canary spermatozoon is evaluated, it is seen that it has a long indented acrosome as well as a rather long flagellum. To determine the morphological structure of canary semen, morphological examination methods used in different poultry can be applied and visually evaluated with different shapes. This study aims to provide information about the comparison and usability of various staining methods used in the morphological examination of canary semen from songbirds. In this study, semen from 12 male Gloster canaries was collected to determine morphological parameters in semen. Collected semen with different morphological evaluation methods; Fixation with 5% formaldehyde, Formalin fixation and Giemsa staining, Giemsa staining, Formalin fixation, and SpermBlue® staining and SpermBlue® staining were evaluated. In the results of the study, while the nucleus was more prominent in Giemsa staining compared to other staining methods used for morphological evaluation, acrosome was observed in SpermBlue® and Giemsa staining. On the other hand, when the sperm fixed with 5% formaldehyde solution were evaluated, it was seen that the acrosome and nucleus were indistinguishable, while the changes in the flagellum were determined much more clearly. As a result of the study, it was reported that the morphological structure of canary semen could be evaluated with all morphological examination methods used.

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1. Introduction

Studies related to infertility problems, reproductive performance, and artificial insemination practices in poultry are generally seen in various exotic songbirds such as sparrows and finches. Studies on canary semen are very limited. Examination of the morphological structure of the sperm and obtaining information about it provide important information about

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the fertile ability and quality of the sperm. Lupold et al. (2019) reported that there is a relationship between the morphological structure and motility of spermatozoa in songbirds. It has been determined that in poultry, the spermatozoon can provide more energy due to the longer middle part and therefore the motility value may be higher. In addition, the morphological features of the winged spermatozoa such as the head, middle part, and the structure of the flagellum and the fertility of the poultry are highly correlated with the success of artificial insemination. (Lupold et al., 2019).

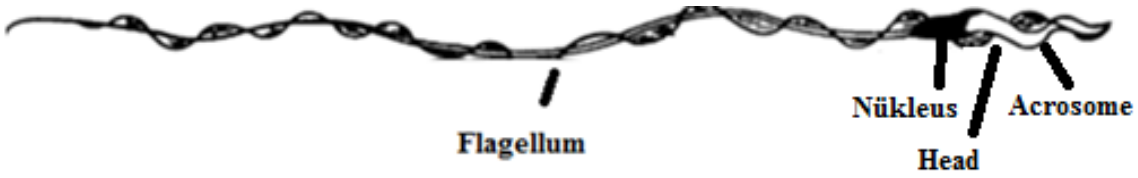

Figure 1. Morphological appearance of the canary spermatozoon (Humphreys, 1972).

The canary spermatozoon has a length of about 300 µm. In addition, the head of winged spermatozoa is considerably larger than that of mammalian spermatozoa (Humphreys, 1972). Thus, the winged spermatozoon has an extremely long flagellum as well as a large acrosome. Due to this morphological structure, poultry semen has some advantages and disadvantages. In addition, this situation directly affects the fertility ability of spermatozoa. In a study, it was emphasized that the long spermatozoon length in birds has a positive effect on the fertilization ability. It has also been reported that there may be a relationship between age and the length of spermatozoa in male birds (Cramer et al., 2020). It is thought that there is a relatively direct ratio between the total length of the spermatozoa and the motility of the sperm. It has been stated that the reason for this situation is that the length of the flagellum affects the pushing ability. In addition, studies have emphasized the importance of mitochondria in the middle part of the spermatozoon. However, it has been reported that the relationship between sperm motility and morphology may be selective in songbirds, so more studies are needed on the subject (Cramer et al., 2021). Detection of defects in spermatozoa morphology in poultry, as in mammals, is very necessary to evaluate reproductive performance. In a study conducted for this purpose, head, middle part, and tail anomalies were examined to determine the abnormal spermatozoa ratio (Scheneider et al., 2018).

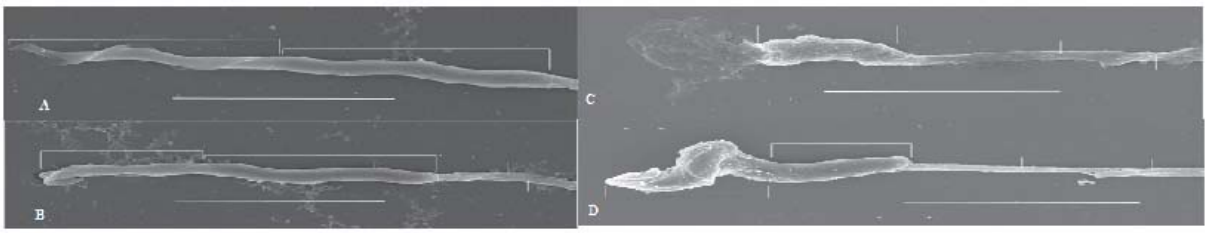

Figure 2. Head-related morphological disorders in winged semen (A: normal acrosome, B: acrosome damage, C: plasma membrane and acrosome deficiency, D: degenerated acrosome) (Hermosell et al., 2013).

2. Materials and Methods

Many methods are used to determine morphological parameters in songbirds. There are very limited resources on the morphology of canary semen. In the study, various staining methods used in the morphological examination of canary sperm, which is one of the songbirds, were evaluated. For this purpose, 12 Gloster male canaries were used in the study. The canaries were selected from young males 1-3 years old. The canaries were kept in production cages measuring 60x50x40 cm against negative effects such as lubrication and stress. Before the semen was collected, the canaries were prepared for semen collection by encouraging sufficient heat generation at the optimum temperature (16-20°C) and with a photoperiodic light application for 15 hours daily.

2.1. Collection of Semen

Cloacal massage method was used to collect semen from canaries. In order to obtain semen from small birds, semen is seen to come out when gently stimulated from the abdomen to the cloacal protuberance located dorsal to the cloaca in male birds. With the application of pressure between the thumb and forefinger of the cloacal protuberance, the semen comes out of the cloaca (Gee et al., 2004). Sperm taken from the cloacal region were collected with the help of a hematocrit capillary tube. Care was taken to avoid contamination while semen was collected. Semen samples contaminated with feces during semen collection were not used in the study.

2.2. Morphological Examination Methods

2.2.1. Detection with 5% Formaldehyde

After the semen sample taken from the canary was fixed with 1 drop of 5% formalin solution, smear was taken on the slide. The slides were left to dry. After drying, it was examined under a light microscope at 40x magnification (Kleven et al., 2009). While the acrosome and nucleus were indistinguishable in the head, it was determined that the flagellum was determined quite clearly.

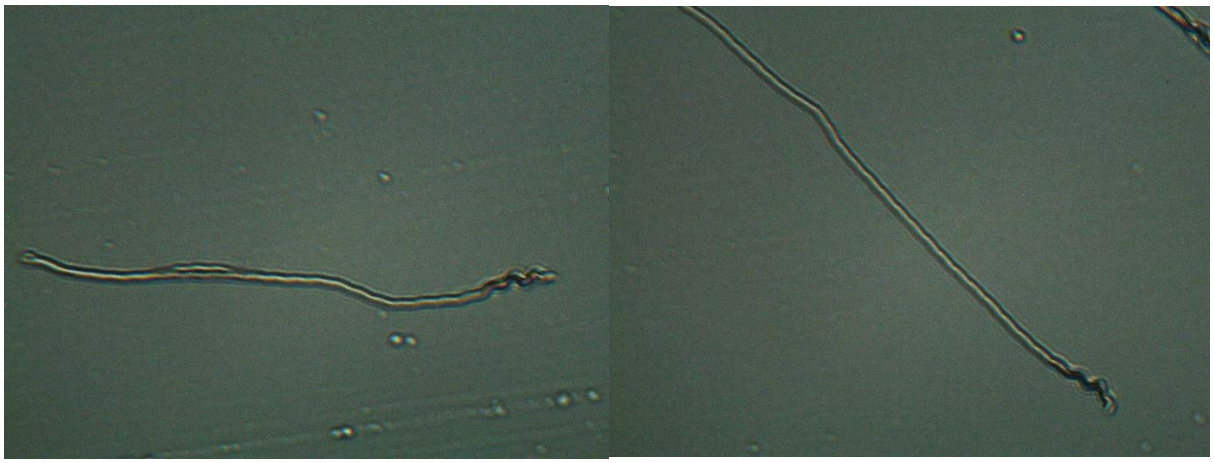

Figure 3. Morphological evaluation of canary spermatozoa with 5% formaldehyde fixation

2.2.2. Formalin Fixation and Giemsa Staining

10 µl of semen sample from the collected semen was fixed with 5% formalin solution. After drying by smearing on the slide, it was painted with Giemsa paint (Camer et al., 2020). After fixation with 5% formolin solution, it was painted with Giemsa dye for 15 minutes. It was evaluated under a light microscope at 40x magnification. evaluated. After the fixation, acrosome and nucleus structures could be distinguished in the head region, while the flagellum was observed to be clearly defined.

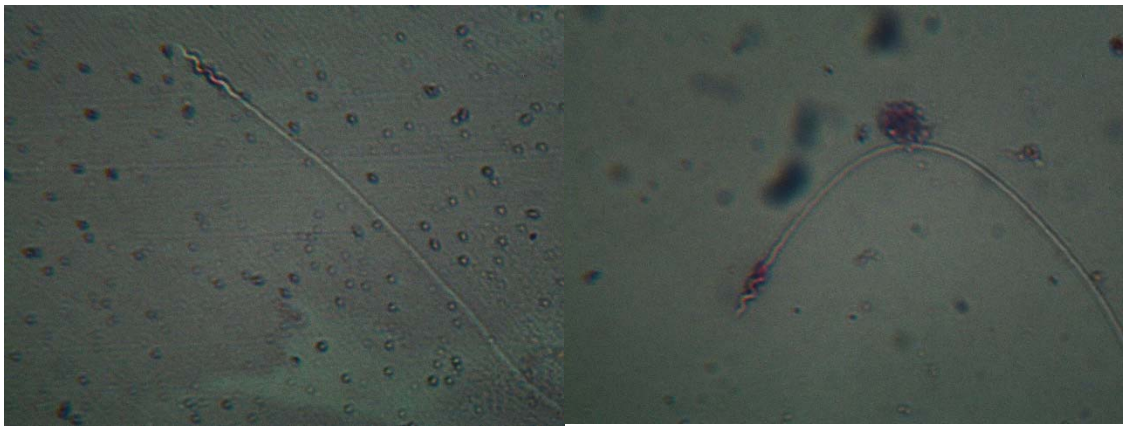

Figure 4. Morphological evaluation of canary spermatozoa by Giemsa staining after 5% formaldehyde fixation

2.2.3. Giemsa Staining

A drop (10 µl) of semen taken by cloacal massage was taken, smeared and dried, and then stained with Giemsa dye for 15 minutes. Afterward, spermatozoa were evaluated under a light microscope at 40x magnification. It was observed that spermatozoa nucleus and acrosome could be distinguished more clearly with this method compared to the Giemsa staining method after fixation with 5% formaldehyde, which is another method. It was determined that the only disadvantage in Giemsa staining was that the paint residue partially blocked the clear vision.

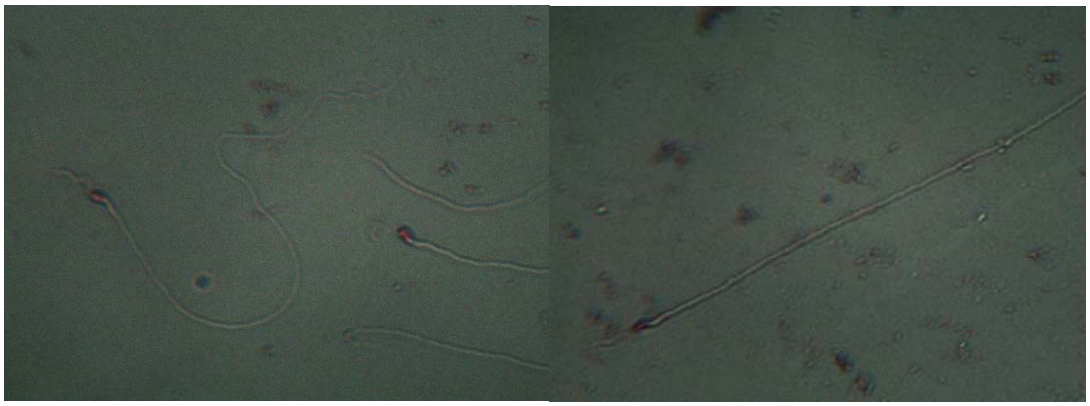

Figure 5. Morphological evaluation of canary spermatozoa by Giemsa staining

2.2.4. SpermBlue® Staining

One drop (10 µl) of the semen taken from the canary was taken, smeared and dried, and then stained with SpermBlue® for 15 minutes. Then, spermatozoa were evaluated under the light microscope at 40x magnification. No fixation was applied before staining. While the

spermatozoon nucleus and acrosome could not be clearly distinguished with this method, it was determined that it was sufficient for the determination of morphological anomalies.

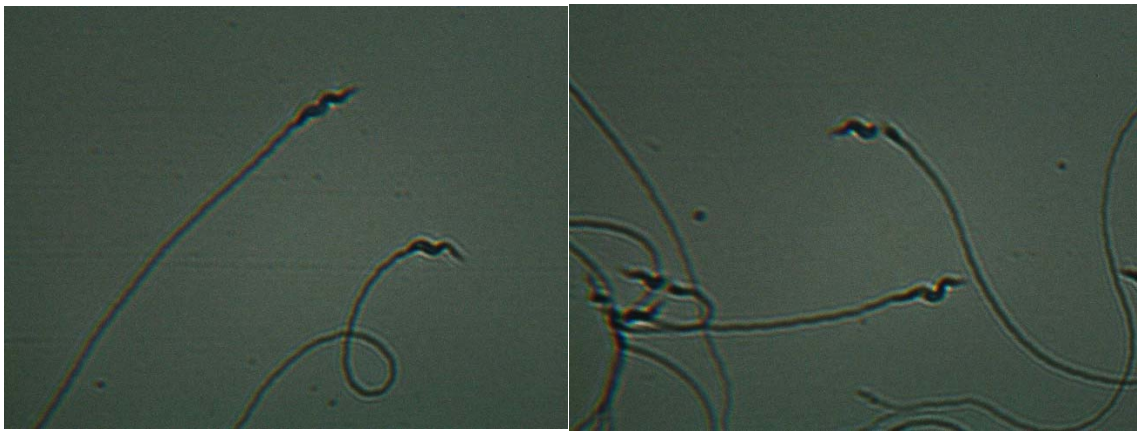

Figure 6. Morphological evaluation of canary spermatozoa by *SpermBlue®* staining

2.2.5. Formalin Fixation and SpermBlue® Staining

The collected semen was fixed with 5% formalin solution and dried by drawing a smear on the slide. Then 15 minutes with SpermBlue® dye. stained (Camer et al., 2020). Then, spermatozoa were evaluated under the light microscope at 40x magnification. After the fixation process, the acrosome and nucleus could be distinguished in the head of the spermatozoon, while the flagellum was determined more clearly and distinctly by SpermBlue® staining without fixation compared to the evaluation method.

Figure 7. Morphological evaluation of canary spermatozoa by *SpermBlue®* staining after 5% formaldehyde detection

3. Result and Discussion

Studies on preserving the qualities of semen in songbirds, improving storage conditions, and evaluating them are increasing. In this sense, it is very important to understand the morphological features of semen and to understand the relationship between semen performance. In this study, besides the different morphological detection methods of canary semen that are frequently used in the field, the effects, evaluation, and advantages and disadvantages of some semen dyes are presented. The current study contributes to this issue since studies on canary semen are very limited and there is not enough data in studies that visually examine the morphological structure of canary semen and semen of close species. It is predicted that the study will be decisive for the use of the appropriate method in the evaluation of spermatozoa morphology in songbird species that differ from mammalian semen and most bird species.

Birkhead et al. (2006), in their study on bullfinch semen morphology, which is a songbird species, determined the semen collected in 5% formaldehyde solution and then stained them with Hoechst 33342 dye and examined them at 100x magnification under a light microscope. Although the morphological staining method overlaps with our study, it does not coincide with the fact that the studies on the subject are generally 40x magnification. In addition, the spermatozoon of some songbird species was handled only as of the head part in the study, and the images were shared as electron microscope images. In another study, the semen collected was fixed in 5% formaldehyde solution and then examined with a light microscope at 16x magnification. Since the morphological appearance was not shared, a comparison with the results of this study could not be made Girndt et al. (2017) observed the semen detected in formaldehyde solution at 40x magnification without staining in order to examine the morphological structure of the semen they received from domestic sparrows in their study. Although the morphological appearance was evaluated in the study, no marking was made to distinguish different regions of the spermatozoon. However, it is reported that the outlines of the spermatozoa are clear and overlap when compared with our current study Helfenshtein et al. (2009), in a study in which they examined the relationship between the morphological structure of semen, motility, and spermatozoon length in domestic sparrows, evaluated the semen detected in 5% formaldehyde solution under a light microscope at 40x magnification after drying it by drawing a smear. Although the results of the study coincide with our current study, a comparison could not be made because there was no visual sharing. The reason why the staining method was not used in the study was explained by the fact that there was no

need for a detailed examination of sparrow spermatozoa. As a result, it is seen that there is not enough data for comparison in the studies examined and the dyeing methods are not used in the studies conducted on songbirds. The results of the study can be evaluated as a preliminary study for researchers and studies related to this subject.

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Research Article

Neuroprotective effect of *Hypericum perforatum* extract against aluminum-maltolate induced toxicity in SH-SY5Y cells

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Abstract

Alzheimer's disease is multi-component neurodegenerative disorder. Oxidative stress disrupts regular functioning of metabolism in early-onset Alzheimer's disease. It causes Tau phosphorylation, formation of neurofibrillary tangle and neuron reduction. Due to intense binding of phosphorylated amino acids to aluminum, it induces self-assembly and deposition of high degree of phosphorylated cytoskeletal proteins, such as microtubule and neurofilament-associated proteins. In this study, it is aimed to consider the antioxidant potential of *Hypericum perforatum* extract against neurotoxicity caused by Aluminum-maltolate ($Al(mal)_3$) and its effects on APP gene expression. Four different groups were determined to observe the impact of *H. perforatum* extract. After the incubation of the cells for 24 hours, only the medium was placed in the first group as control. 500 µM $Al(mal)_3$ was added to the second group of cells. 20 µg mL^{-1} *Hypericum perforatum* extract was added to the third group. For the fourth group, 20 µg mL^{-1} *Hypericum perforatum* extract and 500 µM $Al(mal)_3$ were added. While $Al(mal)_3$ increased total antioxidant status levels in SH-SY5Y human neuroblastoma cells, *H. perforatum* extract significantly inhibited $Al(mal)_3$ induced oxidative stress. On the other hand, *H. perforatum* extract significantly decreased APP gene expression levels depending on $Al(mal)_3$ toxicity in SH-SY5Y cells. According to these results, *H. perforatum* extract significantly inhibited $Al(mal)_3$ neurotoxicity against SH-SY5Y cells. To determine

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synergistic and antagonistic effects of *H. perforatum* extract content is important to examine their specific effects of together with hyperforin, which is a phytochemical produced by some of the members of the plant genus Hypericum, to discover new therapeutic agents against neurodegeneration.

1. Introduction

Alzheimer's disease (AD) is multifactorial chronically progressive neurodegenerative disease (Atasever-Arslan et al., 2020; Ozer et al., 2020). Dementia affects approximately 50 million individuals in worldwide and it is estimated by researchers that this number will reach another 152 million individuals by 2050 (Fleszar et al., 2019). Two main neuropathological features of this disease are β-Amyloid plaques (Aβ) formed by extracellular aggregation of Aβ and construction of neurofibrillary tangles (NFY) by hyperphosphorylated Tau protein (Guan et al., 2021; Vaz and Silvestre, 2020). Aβ aggregation causes lipid peroxidation and apoptosis in neurons (Abdpour et al., 2021).

Although neurotoxic impacts of Aluminum (Al) in the central nervous system are well known, its effect in aluminum-induced neuronal fate is yet uncertain (Exley, 1999; Rebai and Djebli, 2008). Several studies in the literature shown an ascending ratio of AD or AD mortality in areas with high quantity of aluminum in the drinking water (Altmann et al., 1999). Aluminum can affect brain processes like axonal transport, phosphorylation or dephosphorylation of proteins, neurotransmitter synthesis, inflammatory responses, synaptic transmission, protein degradation, and gene expression (Kawahara and Kato-Negishi, 2011). Binding of aluminum to highly phosphorylated cytoskeleton proteins leads to their self-aggregation and accumulation (Muma and Singer, 1996). Maltolate (Mal) is a general ingredient of the human nutrition found in baked grains, soybeans, coffee, and, caramelized and browned foods. Aluminum-maltolate ($Al(mal)_3$) is a potent inducer of apoptosis that has been widely reported as an etiologic factor in AD (Menzies et al., 2017).

Hypericum perforatum plant, with a prevalent distribution around the world, belongs to the order Malpighales, contains more than 16,000 species (Rizzo et al., 2020). It has been widely used for conventional medicine for the treatment of some kind of illnesses, like minor burns, anxiety, and depression. In the literature, there are researches on the chemical structure, pharmacological effects and drug interactions of *H. perforatum* extract and its components. It is known that *H. perforatum* extract and active ingredients of major directly affect

neuroprotective mechanisms and indirectly affect antioxidant mechanisms (Oliveira et al., 2016).

The SH-SY5Y (ATCC® CRL-2266™) cell line, originating from malignant neuroblastoma and showing epithelial morphology (Kitlinska, 2007), was chosen as the cell line to be used to investigate the effects of *H. perforatum* on neurodegeneration induced with $Al(mal)_3$.

The amyloid precursor protein (APP) is a type I single pass transmembrane protein (Collin *et al.*, 2004) which plays an important role in the pathogenesis AD (Aydin *et al.*, 2012).

In this research, it was aimed to determine the antioxidant potential of *H. perforatum* extract towards neurotoxicity which caused by $Al(mal)_3$ and its effects on APP gene expression.

2. Material and Methods

2.1. Preparation of Hypericum perforatum Extract

The flower parts of the *H. perforatum* plant was used for the extract. Plants have been collected from Ege University, Bornova Campus, Izmir Province, Turkey. Identification of species was done by Assistant Professor Ademi Fahri Pirhan, from Ege University, Faculty of Science, Department of Biology. The flowers were dried at room temperature. The extraction method suggested by Deveci et al., 2021 was developed and used (Deveci et al., 2021). Then dried flowers were extracted using a Soxhlet apparatus with methanol. Methanol extracts were dissolved in distilled water using an ultrasonic bath and filtered. Filtration was made acidic with 3% H_2SO_4 at pH 3-4 and extracted with chloroform. The prepared chloroform extracts were dried over anhydrite Na_2SO_4 filtered and concentrated under vacuum.

2.2. SH-SY5Y human neuroblastoma cells

SH-SY5Y cells were subjected to analyze effects of *H. perforatum* on neurodegeneration induced with $Al(mal)_3$. The cells were purchased from "American Type Culture Collection (ATTC)". SH-SY5Y cells were incubated and resuspended according to the research of Arslan et. al., 2017 (Arslan et al., 2017).

2.3. Cell Viability Assay

MTT ("3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide") cytotoxicity assay was performed to detect the suitable dose range of the prepared *H. perforatum* for experiments on SH-SY5Y cells (Arslan et al., 2017).

SH-SY5Y cells were counted and adjusted to 100,000 cells per mL. Ten µL of 6 different stock solutions (1000, 500, 200, 100, 50, and 10 µg mL^{-1}) of the extract were mixed with 90 µL of medium including cells to make the final concentrations as 100, 50, 20, 10, 5, and 1 µg ml^{-1}. Plates were incubated for 48 hours in a humidified environment at 37˚C in a 5% CO_2 incubator. After incubation 10 µL MTT (5 mg mL^{-1}) was added to each well for 4 hours. Then, 80 µL of the supernatant in the wells was withdrawn and 100 µL of 50% solution of sodium dodecyl sulfate (SDS) dissolved in isopropyl alcohol was added (pH 5.5). The prepared SDS mixture destroys the formazan crystals formed by MTT. The resultant color was measured at 570 nm on Multiskan™ GO Microplate Spectrophotometer. As controls, the cells incubated only with the medium were used. The cell viability effect of the extract was compared to control and was calculated (Kaya et al., 2016).

2.4. Preparation of Aluminum Maltolate

$Al(mal)_3$ used in this study is preferable to other aluminum salt forms used in in vitro mechanical studies since it does not form $Al(OH)_3$ precipitates that are insoluble at physiological pH (Zhou and Yokel, 2005). Aluminum was used as a water-soluble mole solution of $AlCl_3.6(H_2O)$. To obtain a 10 mM solution, 20 mM $AlCl_3.6(H_2O)$ was dissolved in distillate water and the pH was arranged to 3.20 mM Maltol (3-Hydroxy-2-Methyl-4-Pyrone) was dissolved in phosphate buffered saline (0.1 M PBS) and the pH was arranged to 7.4. An aluminum-maltol mixture was prepared by combining equal volumes of 20 mM stock aluminum and maltol solutions to give a final concentration of 10 mM for each substance. Then the pH was arranged to 7 with NaOH (Langui et al., 1990).

2.5. Experimental Groups

Four different groups were determined to observe the effect of *H. perforatum* extract. After the cells were incubated for 24 hours, only the medium was placed in the first group as control. 500 µM $Al(mal)_3$ was added to the second group. 20 µg mL^{-1} *H. perforatum* extract was added to the third group. For the fourth group, 20 µg mL^{-1} *H. perforatum* extract and 500 µM $Al(mal)_3$

were added. After 24 hours, RNA isolations of all groups were made for Real-Time PCR (qPCR) experiments.

2.6. RNA Isolation

RNA isolation was performed by using "RNeasy-Mini kit Qiagen Part 1 (74104)" according to the instructions of manufacturer. After concentrations of the RNAs were measured, they was stored at -80˚C.

2.7. Real Time PCR

Analysis of the expression of APP, and β-Actin genes was performed with the "GoTaq 1-Step RT-qPCR System Technical Manual (Cat no: A6020)" kit. β-Actin gene expression in cells was examined for normalization. To calculate alterations of gene expressions was used 2−ΔΔCT method (Livak and Schmittgen, 2001).

2.8. Total Antioxidant Status (TOS) Measurement

Total Antioxidant Status (TOS) levels of the experimental groups were measured a "Rel Assay Diagnostics (Cat no: RL0017, Gaziantep, Turkey)". The kit method is based on the reduction of the green-blue ABTS radical to the colorless ABTS form by antioxidant molecules in the sample. The total antioxidant level in the sample was measured at 660 nm absorbance. The assay was calibrated with an antioxidant standard solution known as Trolox equivalent, which is a vitamin E analog. The results obtained are expressed in mmol L^{-1}. TOS amount was calculated according to the formula:

$$\text{TOS amount} = \frac{[\Delta\text{Abs H2O} - \Delta\text{Abs Sample}]}{[\Delta\text{abs H2O} - \Delta\text{Abs Standart}]}$$

2.9.Total Oxidant Status (TAS) Measurement

Total Oxidant Status (TAS) level measurement was made with a "Rel Assay Diagnostics (Cat no: RL0024, Gaziantep, Turkey)". The experimental protocol is based on the oxidant molecules in the sample oxidize the iron ion-chelate complex to iron ion. The oxidation reaction is prolonged by the booster molecules present in large quantities in the reaction medium. In this

way, the ferric ion forms a colored complex with the chromogen in an acidic environment. The color intensity is related to the total amount of oxidant molecules present in the sample. An it can be measured spectrophotometrically at 530 nm absorbance The assay was calibrated with hydrogen peroxide standart solution. The results obtained are expressed in μmol L^{-1}.

$$\text{TAS amount} = \frac{[\Delta\text{Abs Sample}]}{[\Delta\text{Abs Standart}]} \text{x } 10^{*}$$

(*= Concentration of standart)

2.10. Statistical Analysis

For statistical analysis of results, "Statistical Product and Service Solutions (SPSS)" program was used. Statistical differences between the groups were analyzed by using "One-way analysis of variance (ANOVA)" in SPSS program and with the "Least significant difference (LSD) test" as post-hoc test.

3. Results and Discussion

Hypericum species generally have high antioxidant properties according to the diversity of phenolic compounds they contain. In regard with the results of a research investigating the in vitro free radical discharge impact of the *H. perforatum* plant, it was determined that the antioxidant effect of the extract was directly proportional to the concentration. It has been stated that the plant extract has strong hydroxyl and superoxide anion discharge impacts and also prohibits lipid peroxidation (Mir et al., 2019).

Hypericum extracts show antidepressant properties through hyperforin. In addition, naphthodiantrone hypericin contained in the extract is a potential anticancer agent and there are studies where it has been identified as a potential treatment against neurodegenerative diseases, such as AD (Garg et al., 2012; Hofrichter et al., 2013). It has been determined that tetrahydrohyperforin increases the intracellular Ca^{+2} concentration, resulting in stronger synaptic responses, which prevents degeneration caused by Aβ oligomers (Ittner and Götz, 2011; Nussbaum et al., 2013).

In another study, it was found that the expression of APP gene increased with the gradual increase of $Al(mal)_3$ dose (Liang et al., 2013). Zhang et al., found that APP gene expression

enhanced in PC12 and PC12-ApoE4 cells treated with $Al(mal)_3$ depending on gradual increase depending on $Al(mal)_3$ concentration (Zhang *et al.,* 2019). Therefore, *H. perforatum* extract may have therapeutic effect on APP gene expression in the neurodegeneration induced by $Al(mal)_3$.

Apart from AD, studies evaluating the effects of hyperforin-rich standardized *H. perforatum* extract on Parkinson's disease are also available in the literature. An extract containing 6% hyperforin was administered intraperitoneally to rats with neurotoxicity at 4 mg kg^{-1} per day. Accordingly, it was determined that there was a decrease in nerve damage in the extract administered group (del Rio et al., 2013).

It has been shown that when 100 and 200 μM of $Al(mal)_3$ is applied to SH-SY5Y cells, there is an excessive increase in intracellular ROS levels and synthesis of proteins involved in neuroinflammation. Also it induces unfolded protein response (UPR). Stress by Endoplasmic reticulum (ER) causes many neurological disorders, including AD. In a study researching the impact of aluminum on ER stress, it was shown that aluminum potentially increases protein amounts such as caspase 3, caspase 9, EIF2α, PERK, and inflammatory biomarkers nitric oxide, HMGB1, NF-Kb and NLRP3. It was also determined that aluminum changed IL6, IL10, IL1β, and TNFα mRNA levels. Comprehensive findings demonstrated that aluminum regulates the UPR influence via ER stress resulting in activation of inflammatory pathway and apoptotic proteins in neuronal cells (Rizvi et al., 2016).

In an animal model research about the neuroprotective effect of *H. perforatum*, they concluded that alcoholic extract has neuroprotective activity, which may be related to the prevention of Aβ peptide neuronal degeneration observed in AD (Akiyama et al., 2000).

In order to understand whether *H. perforatum* extract has a protective effect against the neurotoxicity of $Al(mal)_3$, the effect of the extract on cell viability in SH-SY5Y cells was investigated. SH-SY5Y cells were incubated with different concentrations as 100, 50, 20, 10, 5, and 1 μg mL^{-1} of *H. perforatum* extract. The most appropriate dose of *H. perforatum* extract for all experiments in this research was determined as 20 μg mL^{-1} considering cell viability (Figure 1).

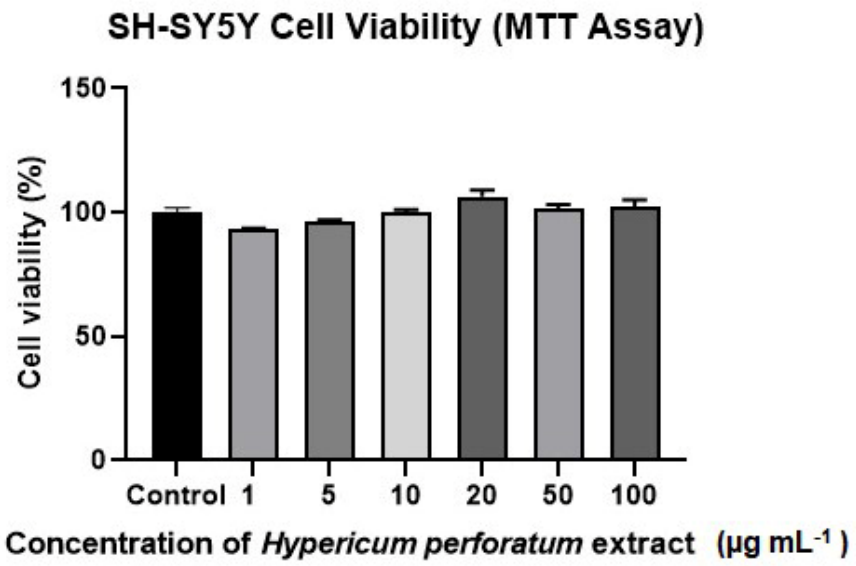

Figure 1. Cell viability % results of *Hypericium perforatum* extract on SH-SY5Y cells.

$Al(mal)_3$ incubation of SH-SY5Y cells was significantly increased APP gene expression in those cells. Conversely, *H. perforatum* extract meaningfully decreased this effect of $Al(mal)_3$ in SH-SY5Y cells. In addition, gene expression level of APP gene is lower than control in the group 2 (only *H. perforatum* extract incubation) (Figure 2).

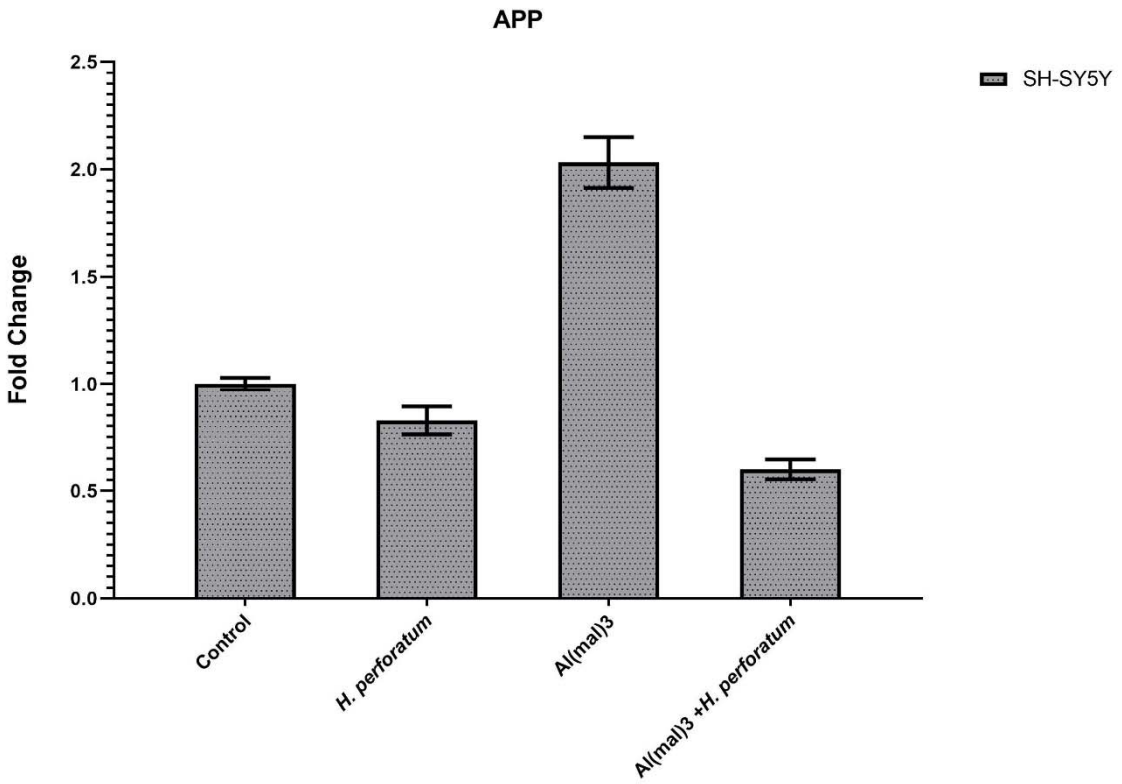

Figure 2. APP gene expression levels in control, H. perforatum, $Al(mal)_3$ and *H. perforatum* + $Al(mal)_3$ treated SH-SY5Y cells.

In this study, $Al(mal)_3$ significantly increased TOS level comparing with other experimental groups and control. Altough TOS levels of *H. perforatum* involved in groups 2 and 4 were higher than control group, it significantly decreased this effect of $Al(mal)_3$ in group 4 (Figure 3). On the other hand, *H. perforatum* and $Al(mal)_3$ did not change TAS levels in SH-SY5Y cells (Figure 4). It was shown in the literature that $Al(mal)_3$ significantly increased TOS level in SH-SY5Y and PC-12 cells (Wang *et al.,* 2019).

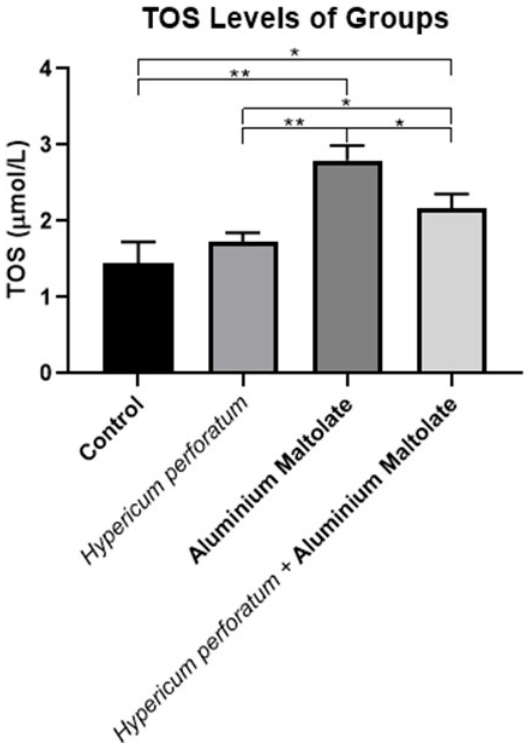

Figure 3. TOS levels in control, *H. perforatum*, $Al(mal)_3$ and *H. perforatum* + $Al(mal)_3$ treated SH-SY5Y cells.

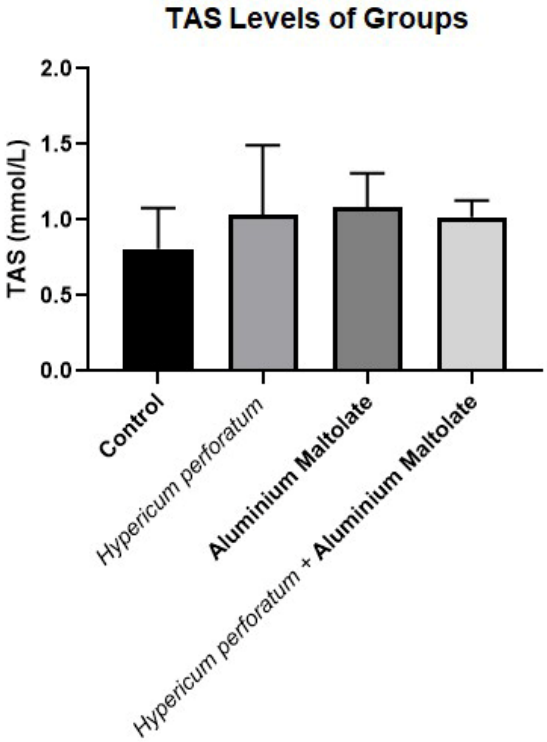

Figure 4. TAS levels in control, *H. perforatum*, $Al(mal)_3$ and *H. perforatum* + $Al(mal)_3$ treated SH-SY5Y cells.

It was shown that hyperforin, the major content of *H. perforatum*, reduces ROS production and prevents the formation of Aβ oligomers and Aβ fibrils in neurotoxicity induced by injecting amyloid fibrils into the hippocampus of rats (Dinamarca et al., 2006; Griffith et al., 2010). Huang et al., showed that hyperforin decreased $Al(mal)_3$ induced Tau phosphorylation and Aβ1-42 formation and increased cell viability. Degradation of APP are regulated by the inhibition of AKT/GSK-3β signaling pathway. It is thought that Hyperforin controls phosphorylation of this signaling pathway (Huang et al., 2017).

According to these results, *H. perforatum* extract significantly inhibited $Al(mal)_3$ neurotoxicity against SH-SY5Y cells. To determine synergistic and antagonistic effects of *H. perforatum* extract content is important to examine their specific effects of together with hyperforin to discover new therapeutic agents against neurodegeneration.

4. Conclusion

Various researches have revealed that *H. perforatum* extract has a neuroprotective effect, but it was shown for the first time in this study that it has a neuroprotective effect against $Al(mal)_3$ toxicity. According to these results, *H. perforatum* extract significantly inhibited $Al(mal)_3$ neurotoxicity against SH-SY5Y cells. To determine synergistic and antagonistic effects of *H. perforatum* extract content is important to examine their specific effects of together with hyperforin to discover new therapeutic agents against neurodegeneration. In the direction of fully elucidate the effect of *H. perforatum* on neurodegeneration, studies with this plant extract should be increased.

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Research Article

Bacteriological-profile of some vegetables sold in Lafia Metropolis, Nasarawa State, Nigeria

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Abstract

The bacteriological-profile of fresh spinach and cabbage sold in Lafia Modern Market were analyzed in the Microbiology laboratory of Nasarawa State Polytechnic, Lafia by homogenizing 1g of the sample in 10 ml of peptone water. An aliquot from a 10-fold serial dilution was inoculated using the pour plate method into different bacteriological medium at 37°C for 24 hours. Total number of colonies was counted and identified using standard procedures. Results indicated that spinach had the highest average bacteria count of 1.4 x10^4CFU/g, 9.2x10^3CFU/g and 6.1x10^3CFU/g Nutrient agar, MacConkey agar and Salmonella-Shigella agar respectively, while cabbage had an average bacterial count of 1.0x10^4CFU/g, 6.0x10^3CFU/g and 4.1x10^3 CFU/g on Nutrient agar, MacConkey agar and Salmonella-Shigella agar respectively. The genera of the bacteria isolates identified were *Streptococcus* spp (8%), *Bacillus subtilis* and *Pseudomonas aeruginosa* (15% each), *Staphylococcus aureus* (16%), *Escherichia coli* and *Salmonella* spp (23% each). These isolated bacteria are of public health importance due to their implication in food borne illnesses. It is recommended that hygiene-level of the entire vegetable processing value chain should be improved upon in other to prevent or reduce bacterial contamination.

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Vegetable,
Cabbage,
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1. Introduction

Vegetables are plants rich in essential bioactive nutrients like minerals, fiber and vitamins (Conner, *et al*., 2017), they are consumed (raw or preheated) by ruminant animals and humans (de Evan, *et al*., 2019). Most vegetables are usually green plants e.g. spinach, lettuce, and pumpkin, while others such as cabbage, onion, mushroom and radish, are non-green vegetable plants (Amao, 2018). Vegetables are very fragile in nature, therefore in order

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not to lose their unique nutrients, it is recommended that consumers eat them raw or slightly-heated, as too much heat resulting from over cooking, will destroy their nutrients (Chaturvedi, *et al.,* 2013; Feng, *et al.,* 2022).

The health benefits derived from consuming fresh vegetables containing high fiber and vitamins content make them more popular for the people who care about boosting their immunity using proper diet especially in the post-COVID pandemic era (Chowdhury, 2020).

Spinach (*Spinacia oleracea*) is an edible fiber-rich, low-calories, flowering vegetable plant belonging to the family *amaranthaceae*, native to central and western Asia (Britannica, 2022; Hedges and Lister, 2007). It grows fastest in well-drained soil rich in organic matter such as compost manure and with a pH of 6.5 to 7 (Warid, 2018). In order to grow spinach twice a year, it is planted about 4 to 6 weeks before the last frost in the spring, and again 6 to 8 weeks before the first frost in the fall andthe plants are spaced 12 inches apart, this gives leaves room to reach full size (Ozlem and Sener, 2005). Spinach is best known for being an extremely rich source of phytochemicals (like lutein, phenolic compounds, zeaxanthin, and β-Carotene) and core nutrients (Hedges and Lister, 2007). The leaves are often used in making various vegetable delicacies, it's main micronutrients is vitamins A (from β-Carotene), C and K, as well as folate and minerals such as calcium, iron and potassium. Other nutrients present in smaller quantities include vitamin E, some B vitamins -Thiamine (B_1), riboflavin (B_2) and the minerals magnesium, manganese and zinc (Athar *et al*., 2004b; Hedges and Lister, 2007).

Cabbage (*Brassica oleraceaver-capitata*) is an important vegetable known to mankind for over 4,000 years (Teshome, *et al*., 2018). It is a member of the mustard or cruciferous family (*Brassicaceae*), which includes mustard, rape, turnip, wasabi (*Eutrema wasabi*), radish, watercress, many oriental vegetables, and a very important model plant *Arabidopsis thaliana* (Shrestha, 2019). In terms of life cycle, cabbage is a short lived perennial crop, usually biennial. Cabbage grows best on well-drained fertilized soils with constant availability of adequate moisture and under moderate temperature and pH in the range 6.0 - 6.5. It is essential not to grow cabbage on the same field year after year because of accumulation of various pathogens, to which crops is highly susceptible (Tsoho and Salau, 2012).

Cabbage contains calcium in the range of 22-150mg/100g. Its accumulated mineral source is at very high level of phosphorus, sulphur, chlorine, calcium, iron and potassium (Jahangir *et al.*, 2009). Cabbage comprises potentially useful amount of copper, zinc and a number of other important minerals and trace elements. Cabbage has a lot of health benefits

which includes prevention of oxidative stress, induction of detoxificative enzymes, and stimulation of immune system reduction of cancer cells and inhabits malignant transformation and carcinogenic mutation. It also plays an important role in the etipathology of many diseases such as vasospasm, atherosclerosis, cancer, heart attack, stroke and liver damage (Athar, *et al.,* 2004a).

Although the consumption of vegetable products has increased in recent years (Feng, *et al.,* 2022), these vegetables have also become vehicles for the transmission of some kinds of pathogens when eaten raw from unhygienic preparation causing food poisoning(Chaturvedi, *et al.,* 2013; Chowdhury, 2020). They are widely exposed to microbial contaminations through contact with water, soil, dust, and by handling at harvest or during post harvest processing. They therefore harbor both human and plant pathogens (Teshome, *et al*., 2018). Pathogenic bacteria that have been detected in fresh vegetables (Spinach and cabbage) are coliform bacteria, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp (Tambekar and Mundhada, 2006).

This study therefore aims to identify and compare the bacteriological load of cabbage and spinach sold in Lafia modern market in Nasarawa State, North-Central Nigeria.

2. Materials and Methods

2.1. Sample Collection

Fresh spinach (*Spinacia oleracea*) and cabbage (*Brassica oleraceaver-capitata*) vegetables were purchased from modern market Lafia, Nasarawa State, North-Central Nigeria.

2.2. Sample Preparation and Dilution

1 grams (1g) of each sample was weighed using a weighing balance, centrifuged and homogenized in 10 ml of peptone water for 10mins. A 10-fold serial dilution was done using 1ml of the homogenate and 9 ml of sterile distilled water in five test tubes.

2.3. Enumeration of Bacterial Dilution

An aliquot was taken from each dilution and was inoculated into the following bacteriological medium (Nutrient agar, MacConkey agar and *Salmonella-Shigella* agar) using the pour plate method, and subsequently incubated at 37^0C for 24hours. The total number of

colonies were counted and differentiated on the basic of their morphology and counts were obtained from different colonies (Oleghe *et al*., 2022).

2.4. Preparation of Pure Culture

Pure cultures of representative's bacteria colonies were obtained by sub-culturing and streaking onto sterile freshly prepared nutrient agar. The plates where then incubated and maintained in agar slants at 37^0C for 24 hours.

2.5 Characterization of Isolate and identification

Identification of isolates was confirmed by Gram staining, cultural, morphological and biochemical characterization using routine laboratory techniques according to Oleghe *et al*., (2020). All analyses were performed in triplicate.

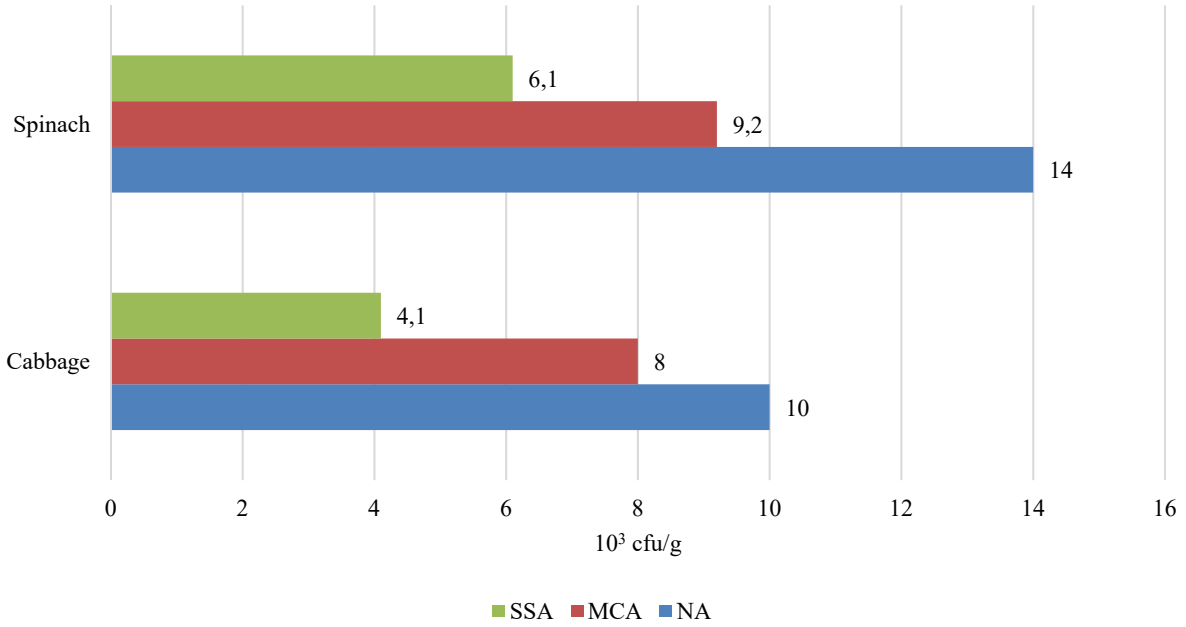
3. Results and Discussion

The results of the plate count indicated that spinach had the highest average bacteria count of 1.4 $x10^4$CFU/g, $9.2x10^3$CFU/g and $6.1x10^3$CFU/g on Nutrient agar, MacConkey agar and *Salmonella-Shigella* agar respectively, while cabbage had an average bacterial count of $1.0x10^4$CFU/g, $6.0x10^3$CFU/g and $4.1x10^3$ CFU/g on Nutrient agar, MacConkey agar and *Salmonella-Shigella* agar respectively (Table 1; Figure 1).

Table 1: Average Total Count of Bacteria Isolate in the Samples on Some Vegetables Sold In Lafia in Colony forming unit per gram (CFU/g).

Sample	***NA***	***MCA***	***SSA***
CIN	1.0×10^4	$8.0x10^3$	$4.1x10^3$
SP	1.4×10^4	$9.2x10^3$	$6.1x10^3$

Key: CIN: Cabbage SP: Spinach, NA: Nutrient agar, MCA: MacConkey agar, SSA: Salmonella Shigella agar



Key: NA: Nutrient agar, MCA: MacConkey agar, SSA: Salmonella Shigella agar

Figure 1: Chart Comparing the Average Bacterial Load from Spinach and Cabbage on the Different Agar used

From the percentage (%) occurrence (figure 2), it shows that *Salmonella* spp and *Escherichia* coli were the most predominant organisms isolated (23%), while *Streptococcus* spp was the least (8%).

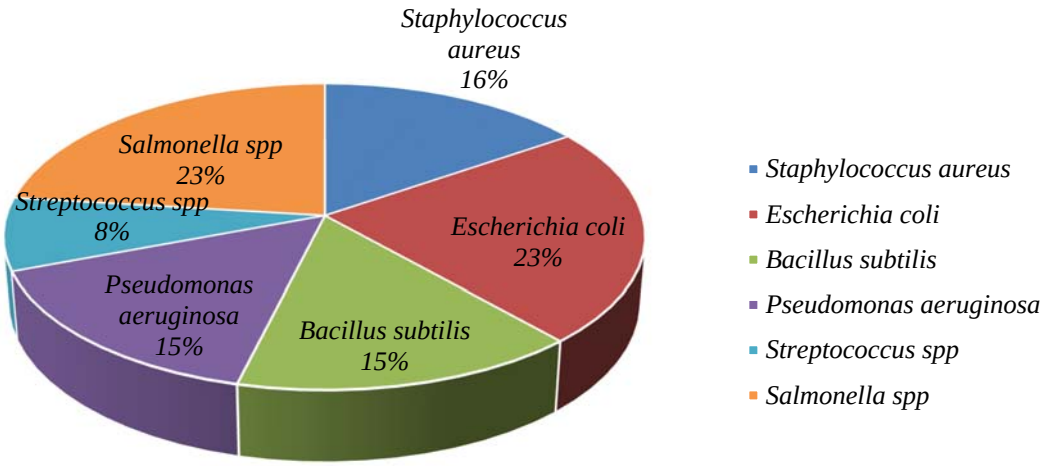


Figure 2: Chart showing the percentage (%) occurrence of the isolates in the samples

In this comparative assessment of the bacteriological content of cabbage and spinach, it was found that the level of bacteria in spinach is higher than that of cabbage. Generally, the bacteria found were similar but in different proportions (Table 3). Bacteria like *Staphylococcus aureus*, *Streptococcus* spp, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella* spp and *Escherichia coli* were found in the samples (Table 2).

Table 2: Showing the Cultural, Morphological and Biochemical characteristics of Bacteria Isolate from Spinach and Cabbage using MacConkey, Nutrient and Salmonella Shigella Agar

Parameter	***Isolate 1***	***Isolate 2***	***Isolate 3***	***Isolate 4***	***Isolate 5***	***Isolate 6***
Cultural characteristics	Dry, circular, Whitish to creamy flat on nutrient agar	Circular pink, elevated, on Mac Conkey agar	Whitish circular colonies on Nutrient agar	Transparent to milky irregular flat on Nutrient agar	Dry, spreading, whitish, flat on nutrient agar	Circular, pink colonies with black center on SSA
Morphologicalcharacteristics	Cocci in clusters	Long rods	Rod	Short rods	Cocci in chains	Rod
Gram reaction	+	-	+	-	+	-
Coagulase	+	-	-	ND	-	ND
Catalase	+	-	+	-	+	+
Indole	-	+	-	+	-	-
Oxidase	-	-	-	-	-	-
Glucose	+	+	+	+	+	+
Maltose	+	+	+	+	+	-
Lactose	-	+	-	-	+	+
Probable bacteria	*Staphylococcus aureus*	*Escherichia coli*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Streptococcus* spp	*Salmonella spp*

Key :+ : positive **--**: negative **ND:** not determined

Table 3: Occurrence of the isolates in the samples

Samples	***Isolates***
Spinach	*Staphylococcusaureus*, *Escherichiacoli*, *Bacillussubtilis*, *Pseudomonasaeruginosa*, *Streptococcus*spp, *Salmonella*spp
Cabbage	*Staphylococcusaureus*, *Escherichiacoli*, *Bacillussubtilis*, *Pseudomonasaeruginosa*, *Salmonella*spp

Staphylococcus aureus is a gram positive bacterium found on the skin or in the nose of both healthy and unhealthy individuals. They come in contact with the vegetables during pre and post harvest practices like hand picking, planting, etc. also in the market where these vegetable are sold there is constant human contacts from both the vendors and consumers (Chaturvedi, *et al.,* 2013). Staphylococcal gastroenteritis is mainly caused by the consumption of food contaminated with *Staphylococcal aureus* strains (Izah *et al.,* 2016). The symptoms of staphylococcal gastroenteritis may include vomiting, abdominal cramps, headache, weakness and fatigue (Akhigbemidu *et al.,* 2015).

Salmonella spp and *Escherichia coli* could contaminate vegetables through fecal contamination of water, hands or /and soil. These pathogens could be from the water of irrigation and from the common unhygienic practices of the vendors. The microbial quality of irrigated water is critical because water contaminated with animal waste can introduce pathogens into vegetable products during pre-harvest and post-harvest activities via direct or indirect contamination. Therefore the bacteriological quality of irrigation water has a paramount importance to the safety of fresh and minimally processed vegetables (Solomon *et al.,* 2002). *Salmonella* and *Escherichia coli* cause varying degrees of intestinal disorders which include diarrhea which is sometimes bloody, urinary tract infection, abdominal cramps and dysentery (Odu and Imaku, 2013).

Bacillus subtilis is a gram-positive, rod-shaped facultatively anaerobic forming bacterium commonly found in soil and food due to preformed heat stable toxins. *Bacillus* in food products at concentrations exceeding 10^4 spores or vegetative cells per gram can cause food poisoning (Ehling-Schultz *et al.,* 2006; Meldrum *et al.,* 2009).

Pseudomonas aeruginosa comes in contact with vegetables through water, fertilizer or use of biocides during cultivation. The contamination of *Pseudomonasaeruginosa* on

vegetable may occur during harvesting, handling, processing and transit. Vegetable may come in contact with some soil, insects and water which they are represented as important sources of contamination in field including runoff water from nearby animal pasture and irrigation from contaminated sources (Chaturvedi, *et al.,* 2013).

4. Conclusion

Contaminated water, fecal materials, unhygienic environment and handling of vegetables and vegetable products by vendors were said to be the main source of contamination of fresh vegetable. The bacteria content of spinach was found to be more than that of the cabbage especially the inner layers of the cabbage because it is covered while spinach is exposed. This study therefore recommends that all those involved in the entire vegetable value chain should improve on their hygiene practice especially when handling or processing vegetables in other to prevent or reduce bacterial contamination to the barest minimum. Government should create portable water irrigation systems for vegetable farming, educate farmers, vendors and end users on the dangers of these pathogens so that more precaution will be taken from pre-planting to post harvesting stages. Also, consumers are advised to thoroughly wash vegetables during processing for consumption.

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Research Article

The effect of artificial insemination on egg fertilization at different times of nest construction in gloster canaries

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Abstract

When canaries are sexually active, they mate during the breeding season. During this time, the female canary builds a nest. In the days following the completion of nest construction, they lay eggs and incubate. In artificial insemination, the skill of the practitioner and knowing the appropriate artificial insemination time increase the chance of success. In the study, 8 male and 8 female Gloster canaries were used. Artificial insemination practices arranged for each canary inseminated at different times were called groups. Each artificial insemination application was made 2 times at the specified times. It was applied just before Group 1 canary nest construction was completed and when nest construction was finished, Group 2 canary when nest construction was started and nest was completed, Group 3 was applied when nesting material was given and nest was completed. Female canaries were immediately inseminated by cloacal method with semen taken from male breeders at different times of the nest building phase. Among group applications, it was observed that the 3rd group application was significantly successful when compared to the others. The aim of this study is to determine the effects of artificial insemination applied at different times of the nest building process on fertilization in canaries.

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Fertilization,
Gloster canary

1. Introduction

With the domestication of canaries for hundreds of years, canaries have been bred for their singing and impressive feather characteristics. The breeding season is between spring and summer. The canaries mate during mate selection shortly before the eggs hatch. The egg-laying period of fertile eggs is 13-14 days (Cartwright, 2000). When the hatchlings are 17-25 days old, the female canary is ready to mate again sexually. They consume a lot of energy during nest building and mating behaviors (Beguin et al., 2006). The suitable temperature for production is between 18°C-24°C (Tamura et al., 2021). Light plays an important role in the

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breeding season. Birds that are in an environment of 10 hours of light and 14 hours of darkness in October should be applied with photoperiod lighting by increasing the amount of light regularly, and 16 hours of light and 8 hours of darkness should be applied until July (Ward et al, 2003).During the breeding season, male canaries show signs of estrus due to the increase in testosterone levels and their singing behavior increases significantly (Voight and Leitner, 2008).

It is necessary to feed the canaries rich in protein to prepare them for the breeding season. In addition, the energy requirement increases during the breeding season (Harper and Skinner, 1998). Although female birds do not consume food during the laying period, they meet the need during this period by using the protein and fat reserves in the body (Houston et al., 1995).

The health of the canaries selected for production is very important for production success. Diseases directly related to reproduction can be seen during the breeding season of canaries. Unfortunately, these diseases can cause serious economic losses during production. Various bacterial infections that can develop in the embryonic period and harm the offspring after hatching, as well as embryonic deaths, may adversely affect the production success. Especially *E. Coli*, *Klebsiella*, *Bacillus*, and *Staphylococcus* species have been detected frequently in canaries (Di Francesco et al., 2018). In addition, parasitic infestations of canaries are a big negative. It has been reported that especially *Dermanyssus gallinae* mite causes restlessness due to itching, inability to sleep, decrease in the number of eggs, dermatitis on the skin, weakening and anemia, and death (Circella et al., 2011).

It is vitally important to reduce the stress as much as possible for artificial insemination applied to increase the success of the production season in canaries. In addition, the quality of the semen used for artificial insemination and the method of application should be sufficient. Since increasing the fertile egg rate is also related to the skill of the practitioner, frequent practice will increase success (Blanco et al., 2009). It has been stated that reproductive success in poultry species is directly related to environmental conditions, adaptability to the environment, species-specific behaviors, and success in artificial insemination practices. In addition, it has been reported that the anatomical structure of the species from which semen is taken or artificial insemination, the semen collection technique, the functional quality of the semen specific to the bird species, and the transmission of the sperm that affects the fertilization ability of the egg will increase the success rate of artificial insemination (Gee et al., 2004).

2. Material and Methods

In this study, egg fertility rate was tried to be determined by insemination of female canary at different stages of nest construction. Canaries were selected from young animals 2 years old. 8 male and 8 female Gloster canaries were used in the study. Male and female canaries have never been combined. Nest construction was encouraged by giving nest material after female canary reproductive functions were active.

2.1 Preparing Canaries for the Breeding Season

Eight male and 8 female canaries were used in the study. The canaries were selected from healthy birds 2 years of age. Male and female canaries were evaluated in 60X50X40 cm production cages in a low-stress environment where they could move freely (Figure 1). Starting from January, the light duration was increased with photoperiod application and it was arranged as 16 hours of light and 8 hours of darkness from April to mid-July (Ward et al, 2003). Ectoparasite treatment was applied to the canaries before the breeding period. For this purpose, local Selamectin was applied (Hahn et al., 2014). In addition, male and female canaries were fed with energy and protein-rich diets as the breeding season approached. It is known that especially female canaries benefit from protein and fat reserves in the body during the incubation period (Houston et al., 1995). During the production season, the ambient temperature was between 18-22°C. It has been stated that the required ambient temperature should be 18-24°C (Tamura et al., 2021).

Figure 1. Canary breeding cages (60x50x40 cm dimensions**)**

2.2. Giving Nest Material to Female Canaries and Incubation Process

Females in the same environment as males, but in separate cages, become sexually active by being influenced by the call of active males. Activated female canaries were encouraged to build nests by giving nest materials for internal nesting and nest building (Figure 2). The nest construction phase was completed in 2-6 days, depending on the willingness of the female. Under normal conditions, mates mate during nest building. When the hatchlings are 17 days old, the female starts the nest preparations again (Beguin et al., 2006). However, since there was no male in the cage of the females in the study, this process took up to 25-30 days. Artificial insemination of breeding females was carried out during the nest construction phase. After 25 days after hatching, the nest and materials were given again. The chicks were separated from their parents when they were 30 days old, as they could become self-sufficient. For each female canary, 3 different artificial insemination practices were carried out at 25-day periods throughout the breeding season. Each insemination application was carried out 2 times at the specified times. The first insemination application (Group 1) just before and after the nest construction is completed, the 2nd insemination application (Group 2), when the canary nest construction is started and the nest building is completed, the 3rd insemination application (Group 3) nest material performed when given and when the nest was completed. Egg controls were performed on the 8th day following ovulation and the fertilization rate was evaluated. Females that failed artificial insemination or whose eggs were empty in the controls were kept in incubation until they completed the incubation period of 13-14 days. The aim here is to consider that early termination of incubation may cause stress or hormonal irregularities and adversely affect the subsequent artificial insemination process. The number of eggs, egg occupancy rate, and hatchability of each female canary were noted.

Figure 2. Female canary building a nest

2.3. Semen Collection from Male Canaries

The cloacal massage method was used to collect semen from songbirds (Kucera and Heidinger 2018). The movement of the bird was restricted by holding the feet in the palm. In addition, a suitable position for the application was created. The massage was applied from the base of the cloaca towards the cloacal protuberance by applying light pressure with the help of the thumb and forefinger in the direction of the tail (Figure 3). However, care should be taken as much as possible. Otherwise, contamination of the semen or injury may result. The massage was continued until semen was seen. As soon as semen appeared in light brown, they were collected with the help of a microhematocrit tube. Before starting the artificial insemination practices, the semen of each male canary was evaluated in terms of motility and abnormal spermatozoa, and it was checked whether there was an individual problem in the birds. It was observed that the motility was over 70% and the rate of abnormal spermatozoa was insignificant.

Figure 3. Artificial insemination and revealing protuberance with cloacal massage technique

2.4. Artificial Insemination

It has been reported that semen used in artificial insemination in poultry can reach only about 1% of semen storage tubules (Brillard, 1993). Although there are many artificial insemination methods in poultry, the most commonly used method is the cloacal and intravaginal method. Although the intravaginal method is more successful, it is difficult to cross the vaginal orifice in small birds such as canaries (Blanco et al., 2009). Therefore, the cloacal method, which is an easier and faster method, was preferred in the study as it may cause undesirable situations. Female birds that are sexually active during the breeding season

shed their abdomen feathers (Blanco et al., 2009). This provided convenience during application (Figure 4A). Before artificial insemination, the female bird was flown into the cage and defecated. Thus, the risk of contamination during the application was minimized. A carefully held female bird was stimulated by an abdominal massage before insemination (Blanco et al., 2009). The semen in the microhematocrit tube collected from the male canary was promptly delivered into the cloaca of the female canary (Figure 4 B).

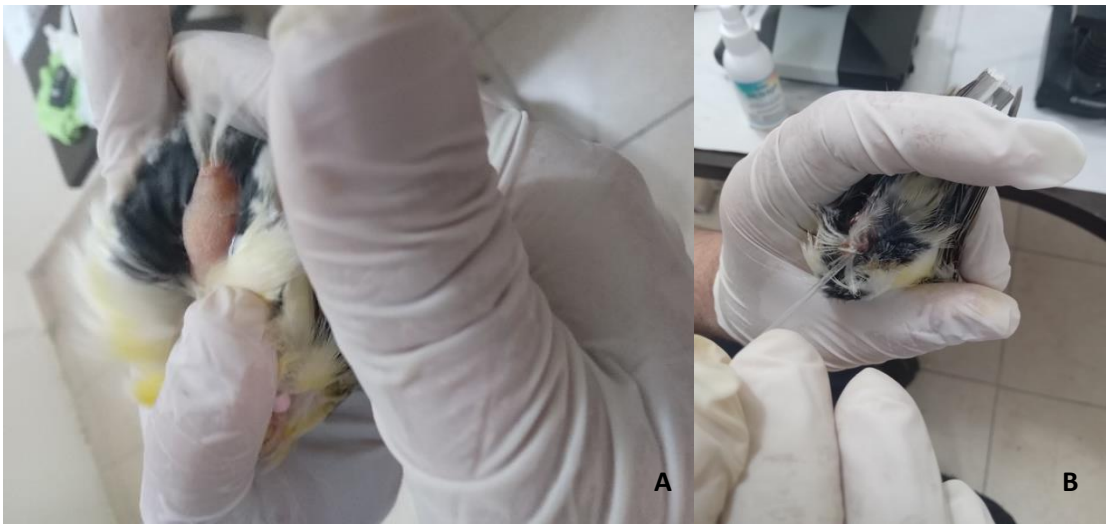

Figure 4. A**:** Active female abdomen feathers shed, B: Artificial insemination technique

2.5. Egg Filling Control and Leaving the Hatchlings

Each female canary was checked for fullness by light on the 8th day following spawning (Figure 5A). Empty eggs were taken and no new nest was given for 25 days for the female to take care of the hatchlings at the end of the incubation period (Figure 5B). It happened in offspring that could not hatch despite fertilization during incubation (Figure 6). Cubs were taken from the female's cage when they were 30 days old. During this time, females were re-seeded according to the procedure established during nest construction.

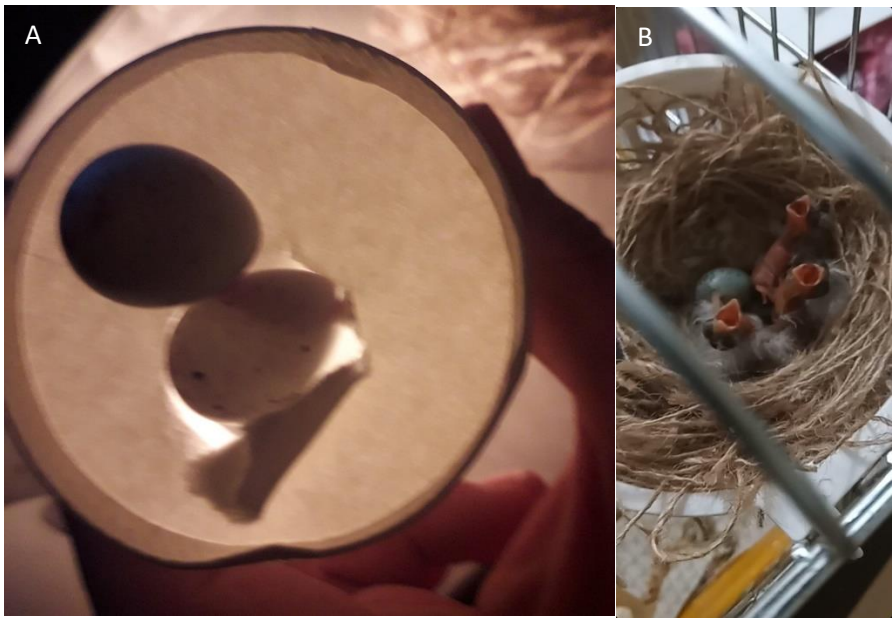

Figure 5. A**:** Empty and full eggs B: Newly hatched chicks

Figure 6. Unable to hatch despite normal development

3. Statistical Analysis

The data obtained in the study were summarized as arithmetic means and standard errors. The statistical significance level for each group used in the study was made with 'One-Way Analysis of Variance'. Duncan's multiple range test was used to investigate the differences between group means. The effects of the groups were evaluated at the $P<0.05$ level. SPSS 20.0 package program was used for variance analysis of the data obtained from the research.

4. Result and Discussion

The canaries have long been widely bred in the world for their beautiful voice and attractive appearance. Spring-summer months are known as the canary breeding season. Some problems that can be seen during mating in canaries may result in low fertility and result in an unsuccessful breeding season for producers. In addition, canaries that are aged or unable to mate due to physical defects, but have quality breeder qualifications, are out of production. Thanks to artificial insemination, negative causes that restrict reproduction can be minimized. However, for this, besides the species-specific breeding conditions, the appropriate artificial insemination time should be known to ensure maximum fertility. In the study, 8 female canaries were inseminated at different times during the nest building phase. Comparing the results regarding the appropriate time for artificial insemination are given in Table 1. There was a statistically significant difference between the groups in terms of egg fertility rates ($P<0.05$). The best fertility rate was found in Group 3. Therefore, according to the results of this study, it can be said that the best insemination time is to be done twice when the nest material is given to the canaries and when the nest construction is completed.

Table 1. Evaluation of egg fertility rate (%) (N=8) (Groups 1, 2, 3: Eggs of inseminated canaries at the beginning, middle and end of nest construction, respectively)

Groups	***Egg Fertility Rate (%)***
Group 1	39.575b
Group 2	29.163b
Group 3	63.538a
SH	5.152
P	**0.013**

a, b: The differences between the means shown with different letters in the same column are significant (P<0.05)

In a study on the subject, it was stated that the most appropriate artificial insemination time for small breed birds should be 3 times 1 week before ovulation (Blanco et al., 2009). In our study, it was observed that the nest building process of canaries lasts 2-6 days and the egg laying process is entered within a few days after the nest construction is completed. Since it is difficult to predict the exact time of ovulation, the nest-building phase has been determined as a guideline. In the study, 2 artificial inseminations were made for each application to provide an example between the groups. In another study, it was stated that the sperm remained in the reproductive canal in chickens for 8-10 days. In addition, it was emphasized that the number of spermatozoa in the reproductive canal was significantly high in 1-6 days (Hemmings et al., 2015). Although the study overlaps with our current study, the fact that the animal material is chicken is seen as a difference. However, the study may answer the question of how long before the time of ovulation artificial insemination should be done in birds. In our study, the best result was when the nest material was given and the nest was completed, but since this process took 2-6 days, it seems that the current study supports our study. Birkhead and Moller (1993) emphasized that semen storage time in poultry is more limited than in reptiles and mammals. However, it has been stated that semen can be stored for at least 8 days in birds. In this respect, the study overlaps with our current study and supports the success of artificial insemination applied when nesting material is given. Since there are not enough resources on artificial insemination in canaries, it is thought that more studies on the subject are needed.

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Research Article

Evaluation of weight loss and some sensory properties in quail eggs coated using different solutions (molasses, molasses + agar, molasses + glycerine, whey)

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Abstract

This study was carried out to determine the effects of different coating materials on weight loss (%) and sensory properties of daily (fresh) quail eggs. For this purpose, quail eggs were coated with molasses, molasses + agar, molasses + glycerine and whey and they were stored at room temperature. No coating material was used in the control group. It was determined that there was a very significant difference between the groups in terms of egg weight loss at all storage times (1st week, 2nd week, 3rd week, 4th week) ($p<0.001$). It was revealed that egg weight loss was lower in all treatment groups compared to the control group. The lowest egg weight loss was observed in the molasses+glycerine group in the 1st, 2nd and 3rd weeks ($p<0.001$), and in the molasses+agar and molasses+glycerine groups at the 4th week ($p<0.001$). When the opinions of the panelists were evaluated, it was determined that the coated eggs were better than the uncoated eggs in terms of appearance, colour and surface smoothness ($p<0.05$). In terms of brightness, control and whey groups were similar, while the other groups had higher scores ($p<0.001$). The opinions of the panelists in terms of adhesiveness, smell and general taste were found to be statistically insignificant ($p>0.05$). When the purchasing attitudes of the panelists were examined, it was determined that the majority (62.5%) preferred molasses + glycerine coated eggs. As a result, it is thought that quail eggs can be coated with molasses, molasses+agar, molasses+glycerin and whey in order to prevent the economic loss caused by the increasing weight loss in parallel with the increase in storage time. Moreover, the fact that the consumption preference is not adversely affected and that some sensory properties are better in coated eggs reveals that quail eggs can be successfully coated with the coating solutions used.

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1. Introduction

A newly laid egg has a sticky layer called the cuticle on its shell (Yüceer, 2013). It is known that this layer, which surrounds the egg, protects the egg from external factors (Wardy et al., 2010) by closing the pores in the egg shell, limits the output of moisture and carbon dioxide (Yüceer, 2013). Therefore, it is important in maintaining egg quality. However, over time, this layer loses its feature (Cansız, 2006). In this case, in parallel with the increase in storage time, carbon dioxide and moisture loss from the pores in the shell occur and weight losses increase. Different coating methods are used to reduce weight loss and maintain egg quality.

There are studies on different coating materials such as beeswax (Mudannayaka et al., 2016; Mudannayaka et al., 2019; Rachtanapun et al., 2021); paraffin (Rachtanapun et al., 2021); chitosan (Ezazi et al., 2021; Caner et al., 2022); propolis (Ezazi et al., 2021; Güler et al., 2022); glycerine (Drabik et al., 2018; Pires et al., 2021); whey protein isolate (Caner and Yüceer 2015), whey protein concentrate (Caner and Yüceer 2015; Pires et al., 2021) in order to increase the quality and shelf life of eggs.

This study was carried out to evaluate some sensory properties (appearance, color, surface smothness, brightness, adhesiveness, smell, general taste, and purchase status of the product) and weight loss (%) of quail eggs coated using different coating solutions.

2. Materials and Methods

2.1. Providing Coating Materials

Molasses, whey, agar and glycerine were used in the preparation of coating solutions in this study. Molasses, a by-product of sugar beet, was obtained from Amasya Sugar Factory. Whey, on the other hand, was supplied as a concentrate from a private Milk and Food Products Industry and Trade Inc. Molasses, which were used as coating material, contains approximately 50% sugar. Moreover, the glycerine used was 87% pure and the Brix value of the whey was 30%.

2.2. Providing Quail Eggs

In this study, Japanese quail (*Coturnix coturnix japonica*) eggs were obtained daily (fresh) from a private quail farm. Eggs in homogenous shape and size were used in the study. Analysis of variance was performed in terms of egg weights and it was determined that there was no statistically significant difference between the groups.

2.3. Preparation of Coating Materials

In this study, a total of 5 groups were formed as control, molasses, molasses+agar, molasses+glycerine and whey. The molasses was diluted to 60% molasses+40% distilled water, while the whey was used as a direct concentrate without dilution. Eggs in the molasses group were coated with diluted molasses (60% molasses+40% distilled water). Eggs in the Molasses+agar group were covered with a solution prepared by adding agar into diluted molasses. Agar was prepared at 1% concentration before mixing with diluted molasses. In order to prevent agglomeration that may occur while dissolving agar in pure water, it was slowly added to the pure water and stirred at 1000 rpm in a magnetic heater mixer to form a solution. Eggs in the molasses+glycerine group were coated with a solution prepared by adding glycerine to the diluted molasses. Furthermore, glycerine was prepared with distilled water at a 3% concentration before mixing with diluted molasses. In the study, agar and glycerine were used as thickeners for better adhesion of the coating material to the shell.

2.4. Coating and Storage of Quail Eggs

In the study, a total of 150 quail eggs, 30 for each group, were used. Eggs were coated with different prepared solutions using the immersion method. Then, the eggs were turned several times to drain the solution remaining on the egg shells and left to dry. It was observed that a film layer formed on the surface of the coated eggs as desired. No coating process was performed in the control group.

Eggs were stored at room temperature for a total of 4 weeks. The temperature and humidity levels of the storage environment were routinely checked and recorded.

2.5. Determination of Egg Weight Loss

In the present study, weight loss was determined according to four different storage periods (1^{st} week, 2^{nd} week, 3^{rd} week, 4^{th} week). Egg weights were determined periodically using a precision scale with a sensitivity of 0.001 g, and the weight loss was calculated as % with the help of the formula below (Bhale et al., 2003).

Egg Weight loss (%) =

[(Starting egg weight) -(Last egg weight)]/ [Starting egg weight] x 100

2.6. Evaluation of Sensory Properties

In order to evaluate the sensory properties of coated and uncoated eggs, randomly selected eggs were submitted to the opinion of 16 people selected among Amasya University Suluova Vocational School Academic Staff. The panellists evaluated the eggs according to different criteria such as appearance, colour, surface smoothness, brightness, adhesiveness, smell,

general taste and attitudes towards purchasing the product in the forms given to them. (According to the hedonic scale, 1: Extremely bad, 2: Very bad, 3: Bad, 4: Below average, above bad, 5: Fair, 6: Below good, above average, 7: Good, 8: Very good, 9: Excellent)

2.7. Statistical Analysis

Analysis of variance of the data obtained from the research was done with one-way ANOVA. Moreover, Duncan's test was used to compare the groups. SPSS (Statistical Package for Social Sciences) 22.0 package program was used for these statistical evaluations (IBM Corp., 2011).

3. Results and Discussion

In this study, weight loss (%) and some sensory properties (appearance, color, surface smoothness, brightness, adhesiveness, smell, general taste, panelists' attitude towards purchasing the product) of quail eggs coated with different solutions (molasses, molasses+agar, molasses+glycerine, whey) were evaluated.

3.1. Evaluation of Weight Loss in Stored Eggs

Weekly weight losses (1st week, 2nd week, 3rd week, 4th week) and general weight losses in quail eggs coated with different solutions are given (Table 1).

It was determined that the coating materials used in quail eggs significantly affected egg weight loss in all weeks ($p<0.001$).

When the egg weight loss after one week of storage was evaluated, it was determined that all the coated groups had lower weight loss than the uncoated (control) group. During this storage period, the lowest egg weight loss was in the molasses+glycerine group ($p<0.001$).

When the weight loss after two weeks of storage was examined, it was determined that the egg weight loss in all the coated groups was lower than the control group ($p<0.001$). Molasses+glycerine was the group with the lowest egg weight loss in two weeks of storage ($p<0.001$).

In the third and fourth weeks of storage, egg weight losses were lower in all coated groups compared to the uncoated control group ($p<0.001$). The group with the lowest egg weight loss in the third week was again molasses + glycerine ($p<0.001$). The groups with the lowest egg weight loss in the fourth week were molasses+agar and molasses+glycerine ($p<0.001$).

Table 1. Effect of coating materials on egg weight loss (%)

Coating	Storage Time (Week)				
	1	**2**	**3**	**4**	**1-4***
Control	1.787[a]	4.221[a]	6.186[a]	8.404[a]	5.149[a]
Molasses	1.447[b]	3.175[b]	4.464[b]	5.773[b]	3.714[b]
Molasses + Agar	1.158[bc]	2.820[bc]	3.757[bc]	4.684[c]	3.105[cd]
Molasses + Glycerine	0.961[c]	2.279[c]	3.120[c]	4.544[c]	2.726[d]
Whey	1.403[b]	3.135[b]	4.305[b]	5.571[bc]	3.604[bc]
SE	0.058	0.111	0.145	0.197	0.095
P	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

a, b, c, d: The averages with different superscripts in the same column differ significantly ($p<0.05$).
SE: Standard Error. *Overall weight loss

According to the previous studies, no study was found that investigated the effects of molasses on egg coating. In this study, it is thought that molasses reduced weight loss because it fills the pores of egg shells like other coating materials.

Most protein-based edible films have been reported to have excellent oxygen barrier properties (Dangaran et al., 2009; Jooyandeh, 2011). Pires et al. (2021) investigated the effects of egg coating on egg internal quality and shelf life with coating solutions prepared with egg whey protein concentrate together with glycerol, sorbitol, and propylene glycol. This study (Pires et al., 2021), which stated that uncoated eggs had higher egg weight losses than the others, is consistent with the results of our study. In another study, Caner and Yüceer (2015), in their study investigating the effects of coating eggs with whey protein isolate and whey protein concentrate on egg quality and egg shelf life, reported that weight loss was higher in uncoated eggs. The results of the current study overlap with the study of Caner and Yüceer (2015). The results of our study are also consistent with other studies investigating the effects of egg coating on egg weight loss with protein-based edible films such as whey protein isolate and whey protein concentrate (Alleoni and Antunes, 2004; Caner, 2005; Davalos-Saucedo et al., 2018; Soares et al., 2021).

Glycerine has plasticizing properties (Eser and Doğruer, 2022). In the present study, glycerine was used together with molasses as a plasticizer.

Drabik et al (2018), in their study investigating the effects of egg coating with glycerine solution on egg quality, emphasized that glycerin reduced egg weight loss and as a result, glycerine can be used in egg storage due to its safe, cheap and easy application.

3.2. Evaluation of Sensory Properties

It is seen that the scores given by the panelists to sensory properties in quail eggs are numerically average of minimum 4,250 (control group; adhesiveness) and average maximum of 8,250 (molasses+glycerine group; brightness) (Table 2).

Table 2. Effect of coating materials on sensory properties

Sensorial Properties	Coating						
	Control	**Molasses**	**Molasses + Agar**	**Molasses + Glycerine**	**Whey**	**SE**	**P**
Appearance	5.500^{c}	6.938^{ab}	7.125^{ab}	7.813^{a}	6.188^{bc}	0.209	**0.004**
Shell Colour	5.563^{b}	7.188^{a}	6.875^{a}	7.500^{a}	6.688^{ab}	0.201	**0.024**
Smoothness	5.250^{b}	6.625^{a}	6.688^{a}	7.375^{a}	6.875^{a}	0.183	**0.003**
Brightness	4.938^{b}	7.375^{a}	7.688^{a}	8.250^{a}	5.875^{b}	0.228	**0.000**
Adhesiveness	4.250	5.875	6.063	5.625	5.063	0.304	0.329
Smell	5.313	5.875	5.813	6.319	6.063	0.185	0.536
Acceptability	5.500	7.063	6.938	7.063	6.375	0.218	0.106

a, b, c: The averages with different superscripts in the same row differ significantly ($p<0.05$).
SE: Standard Error. Maximum: 9 points (excellent), Minimum: 1 point (extremely bad)

While the effects of coating quail eggs with molasses, molasses+agar, molasses+glycerin and whey on appearance ($p<0.05$), color ($p<0.05$), surface smoothness ($p<0.05$) and brightness ($p<0.001$) were significant, the effects on adhesiveness and smell were found to be insignificant ($p>0.05$). Besides, it was determined that there was no significant difference between the groups in terms of general taste ($p>0.05$).

According to the panelists' opinions, it was determined that the egg with the best appearance among the coated and uncoated quail eggs was molasses + glycerine. The groups that had the highest scores in terms of color and brightness were molasses, molasses+agar and molasses+glycerine coated groups. The groups that received the lowest score from the panelists in terms of surface smoothness were the control group.

When the attitudes of the panelists to buy coated eggs were examined, it was seen that the product that 10 out of 16 people (62.5%) marked as "I would definitely buy" was molasses + glycerine coated eggs (Table 3).

Table 3. Attitudes of the Panelists (Consumers) to purchase the product

Coating	Approach to Product					
	1*		2**		3***	
	N	%	N	%	N	%
Control	4	25.00	10	62.50	2	12.50
Molasses	9	56.25	6	37.50	1	6.25
Molasses + Agar	9	56.25	6	37.50	1	6.25
Molasses + Glycerine	10	62.50	4	25.00	2	12.50
Whey	7	43.75	7	43.75	2	12.50

* I would definitely buy, ** Maybe I would, *** I would definitely not buy

4. Conclusion

In parallel with the increase in the storage period, carbon dioxide and moisture loss occurs from the pores in the egg shell, and weight loss increases accordingly. Different coating methods and materials are being researched to reduce weight loss and maintain egg quality. Molasses, molasses+agar, molasses+glycerin and whey were used as coating material in this study. It was observed that the weight loss of quail eggs coated with these solutions decreased significantly during the 4-week storage period (1st week, 2nd week, 3rd week, 4th week). Considering the opinions of the panelists, the fact that consumption preferences are not adversely affected and that some important sensory properties are better in coated quail eggs supports the result of the study positively.

Therefore, it was determined that whey, which is a by-product of dairy products, and molasses, which is a by-product of sugar beet, can be used successfully as coating material. Besides, it was revealed that the use of molasses together with agar and glycerine gave better results in some parameters in the scope of the study compared to the use of molasses alone as a coating material.

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