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African Journal of Biotechnology

Full Length Research Paper

Assessment of water requirement for rice crop at Cyabayaga wetland in Nyagatare District, Eastern Province, Rwanda

Valens Nkundabashaka[1], Sylvestre Habimana[2*], Rose Niyonkuru[1], Claude Kayijuka[1], Antoine Karangwa[2], Saidi Rumanzi Mbaraka[2], Fabrice Rwalinda Musana[2], Eularie Mutamuliza[2] and Marie Chantal Mutimawurugo[2]

[1]School of Agriculture Engineering, College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda, Rwanda.
[2]School of Agriculture and Food Sciences, College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda, Rwanda.

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The overall objective of this present research is assessment of rice-water requirement in Cyabayaga wetland of Nyagatare District in Rwanda. Specific objectives of this research project were to collect and analyze weather parameters, of soil physical properties, crop-water requirements and to recommend efficient water management of the study area. Research design, data collection and field observations were made during July, 2019 to January, 2020. The experiment was carried out in Cyabayaga marshland and was especially based on the assessment of rice water requirement, weather parameters and hydraulic parameters like measurement of soil infiltration rate, soil hydraulic conductivity, flow discharge in irrigation channels, water loss, conveyance efficiency and application efficiency. Evapotranspiration and irrigation needs were determined by Cropwat 8.0 software. Conveyance efficiency (Ec) was found to be 70.05% and 30% of water was lost through seepage and evapotranspiration through weeds in unlined channels. The overall deep percolation, seepage and evapotranspiration losses were found to be 25 mm/day. Considering the highest potential crop evapotranspiration (ETc) of 4.8 mm, it was indicated that huge water loss of 20.2 mm/day was caused by seepage and percolation within the soil. The application efficiency (Ea) was found to be 23.8%. Hence, the overall efficiency was 16.7%. With the current rice yield of 4.25 t/ha, a low water-use-efficiency of 0.089 kg/m^3 was found.

Key words: Cropwater requirements, rice, irrigation requirements, water use efficiency and Cropwat.

INTRODUCTION

Rwanda is land-locked country occupied by the total population of 12.4 million. It has the area of 26,338 km^2. It is among the most highly populated countries of 498.7 persons/km^2 in the region of sub-Saharan Africa. The

*Corresponding author. E-mail: shabimana@gmail.com. Tel: +250 788486031.

population growth rate in the year 2018 was 2.6% (World Bank, 2019). The country is characterized by a hilly landscape and undulated topography. Rice is a key cereal among the important foods in Rwanda with an average yield of 5.25 t/ha and is planted in two planting seasons per year. Season A starts from June to July and ends in Mid-November to Mid-December while Season B starts from Mid-December to July and ends in May to June (Ghins and Karl, 2018).

Marshlands are a potential source for agriculture development and the Government of Rwanda has taken projects of irrigation infrastructure development for increasing production and productivity of crops. Rice is the one of the most vital crops full-grown in developed marshland. The entire surface area of the wetland in Rwanda is roughly 278,000 ha (Léon, 2012) of which an area of 126,441 ha is under intensive agriculture while 4,7224 ha is uncultivated protected marshland (NISR, 2019).

The required range of temperature for maximum growth of the most of the agricultural plants is between 15 and 40°C and the relative humidity suitable for most of the crop plants is about to be 40 to 60%. The wind movement suitable for more crops is about 4 to 6 km/h or 96 to 144 km/day (Arshad et al., 2019).

The higher water retention capacity of particular soil determines its capability to hold a huge amount of water. Capacity of water retention of a given soil depends on the soil characteristics like organic matter, soil texture, structure and depth (Fontanier et al., 2020).

The amount of water required by a crop in a given period of time to reach its normal growth under field environment is called crop water requirements. Crop water requirement comprises of the water losses through evapotranspiration, irrigation and land preparation (Doorenbos and Pruitt, 1977; Eva et al., 2019).

About 95% of the lands are irrigated with the surface irrigation in sub-Saharan Africa and 40% of the supplied water could not reach within the land, only 60 to 70% for application efficiency due to water that is lost along the conveyance system (Salman et al., 2019).

The water loss in a given soil takes place through seepage and percolation in the soil, evapotranspiration from the canopy of the crops and runoff from the field. Evapotranspiration (ET) is high during the planting periods in which 100% of ET comes from evaporation and about 90% of ET comes from transpiration at full crop canopy. Percolation stands for vertical flow of water; the root zone to the water level, while seepage represents groundwater between fields. Percolation and seepage occur at the same time and are hard to separate, which is why they are still considered together (Puasa et al., 2010).

Justification of taking the present research

Cyabayaga wetland was developed to grow paddies in Nyagatare district. The water of Walfu River is diverted through a barrage to an artificial reservoir supplying water into irrigating paddy fields. The marshland has been facing enormous problems due to natural and anthropogenic causes. The marshland is normally affected by insufficient water during dry seasons and excess water during rainy seasons. Climate change is also affecting irregularity in rainfall distribution and amount during cropping seasons, undulating topography in catchment areas that results in heavy soil erosion and sediment transport during the rainy season, which affects the irrigation and drainage channel capacity within the irrigation command. Poor management of irrigation infrastructure and water management also causes poor water use efficiency. The heavy floods during rainy season caused by torrential rains sometimes damage the crop; therefore, a need to find out appropriate solutions is inevitable.

The present research dealt with determination of weather data analysis, soil properties and crop water requirement. The study provides the reasons for poor water and land management in command as well as catchment areas and low water use efficiency. This helps to provide appropriate recommendations for efficient water management and optimum rice production in command area and land use management for catchment area.

METHODOLOGY

Site selection

The marshland of Cyabayaga is positioned in Nyagatare District at the coordinates of 1°25' 15" S, 30°17”38" E. It is situated in agro-ecological zone of in the Eastern Savana with the altitude of 1400 m. The Cyabayaga watershed includes the following sectors Nyagatare, Rukomo, Mukama and Mimuli. The marshland has received and diverted water from Walfu River in Mimuli sector (Karungeri site). During 1970, the hillsides around the wetland were converted into farming and settlement areas. In 2003, the Rural Sector Support Project 1(RSSP1) developed Cyabayaga wetland and thus became useful for growing paddy (Ingabire et al., 2013).

Materials used

During data collection and analysis, the following materials were used: soil sampler to collect soil samples from field to laboratory for soil properties analysis, laptop for data storage and processing, soil lab materials and Cropwat 8.0 software to predict rice water requirement.

Weather data collection and analysis

The meteorological data of Nyagatare station were collected from meteorological agency headquarters for the period of 37 years (from 1982 to 2017): data of temperatures, windspeed, rainfalls, sunshine hours, and relative humidity. Based on analysis of collected weather data, the average values and range cumulative data if needed were determined.

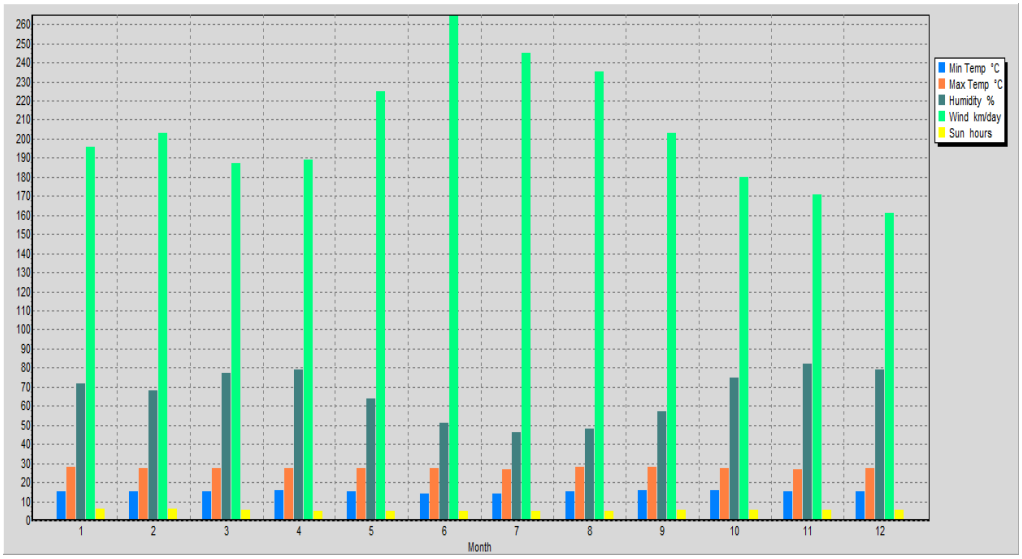

Figure 1. Climatic data from 1982-2017.

Soil sampling and analysis

Soil sample was collected from three separate plots 1, 2 and 3 located in upper, middle, and lower reach in the study area at three different depths: 1) 0-20 cm, 2) 20-40 cm, 3) 40-60 cm and taken into the laboratory of College of Agriculture, Animal Sciences and Veterinary Medicine (CAVM) to analyze its texture, bulk density, infiltration rate and hydraulic conductivity. Based on the recommended procedures, the percentage of sand, silt and clay was determined using Hydrometer methods; hence, USDA-Textural triangle was used to determine particle classes. The soils of experimental sites comprised of sandy loam at the top layer with clay loam beneath. The average bulk density of the Cyabayaga wetland was found to be 1.3 g/cm^3 and it is defining the ideal root growth. The basic infiltration rate was found at 1.0 cm/h. This infiltration rate of the area falls under moderately slow infiltration rate. The average hydraulic conductivity of soils in the study area are highly permeable with 12.96 cm/h (Rapid category) leading to enormous water losses.

Crop water requirements

Cropwat8.0 was used to calculate crop water requirements using input data such as climatic and crop and soil data. In the software, the default irrigation efficiency is 70% as in most surface irrigation systems; the best irrigation efficiency may be achieved at 70%. In this study, 70% efficiency was fed and later compared to the real water used to measure water losses in the field. The crop evapotranspiration received from Cropwat8.0 was used for total water used to harvest the crop after adding deep percolation losses found through stake and nail method.

RESULTS AND DISCUSSION

Weather data collection and analysis

The weather data collected were analyzed on a monthly basis. The meteorological data viz. sunshine hours, wind speed, relative humidity, and minimum and maximum temperature are presented in Figure 1.

The data in Figure 1 show that the average minimum temperature varies between 13.9 and 15.6°C. The highest average minimum temperature of 15.6°C was found in the Month of April, while the lowest average minimum temperature of 13.9°C was found in months of June and July. The highest average temperature of 28°C was found in the months of August and September and the lowest maximum temperature of 27°C was found in the month of July and November. The results show that the warmest month was August and September and the coldest month was found to be June and July. It is evident that average monthly temperature variation is not much.

The highest relative humidity is found during November, with 82% followed by April (79%), December (79%) and March (77%). This is due to their occurrence during rainy season while the lowest relative humidity of 46% was found in the Month of July followed by August (48%), June (51%) as these months fall during dry season. It is obvious that the rainy month seasons present a high amount of relative humidity.

The highest wind velocity of 11.0 km/h and lowest wind velocity of 7.1 km/h was found in the months of July and November, respectively; while the value of 8.5 km/h was the average monthly wind velocity. The range of wind speeds varies from 6.7 to 11.0 km/h with a difference of 4.3 km/h; and it is clear that there is a considerable change in wind velocity throughout the year.

The yellow bars on the Figure 1 represent the trend of sunshine hours, it is evident that the highest sunshine duration is in the month of July and August with 6.7 and 6.5, respectively. These months fall during long dry season. The lowest sun shine hour was found during

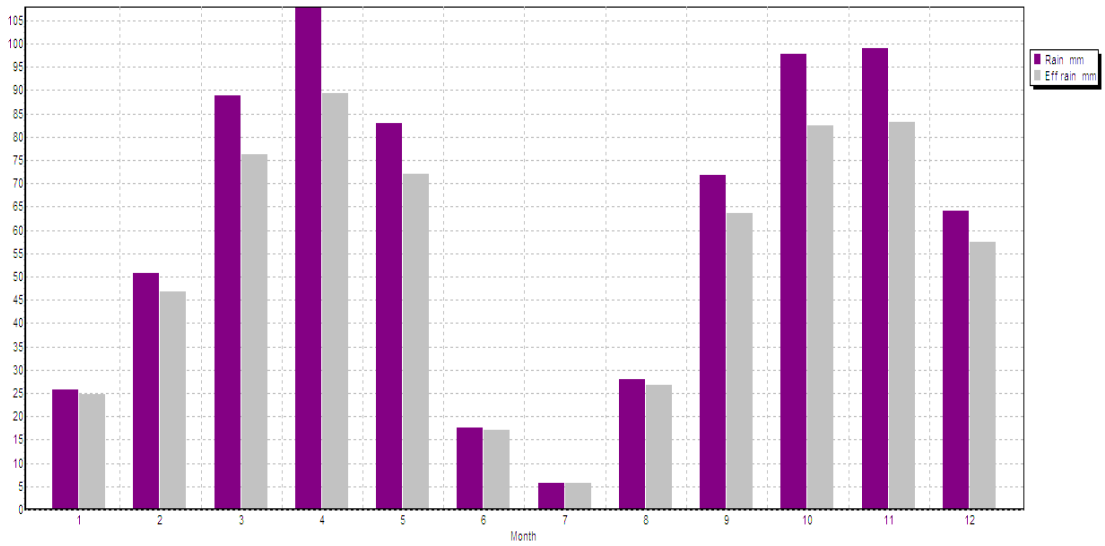

Figure 2. Rainfall and effective rainfall for Cyabayaga**.**

April with 5 h followed by March and February with 5.2 h each. The annual daily average sunshine hours was calculated to be 5.8 h.

Monthly rainfall and effective rainfall

The rainfall data of Nyagatare station were collected from meteorological agency headquarters for the period of 36 years (from 1982 to 2017). Based on analysis of collected rainfall data, the average values, and range cumulative data if needed were determined. The results are presented in Figure 2.

As presented in Figure 2, the month of April received the highest rainfall of 107.9 mm, and July received the lowest rainfall of 5.8 mm with a huge difference of 102.1 mm. The average monthly rainfall was from March to May and was computed to be 93.26 mm while the average rainfall was from September to December was 83.15 mm. The month of April received the maximum effective rainfall of 89.3 mm, while the lowest was obtained throughout July, with the value of 5.7 mm. It is clear that July is the driest month throughout the year where irrigation is highly required. The highest rainfall was obtained in the Month of April and the driest period begins from June to August. The rainy period begins from March to May and from September to December.

Determination of reference evapotranspiration (ETo) by Cropwat

The analysis of inputs climatic data was done using Cropwat 8.0 application that helped to produce the average value of reference evapotranspiration per day (ETo) during each month of the year as is shown in Figure 3.

From Figure 3, it is evident that the maximum ETo was found in the month of August with the value of 5.14 mm/day, followed by July with the value of 5.0 mm/day. The reason for high ETo is because of the dry season months with low RH, comparatively higher wind velocity and more sunshine. It was also found that the lowest ETo is 3.64 mm/day during November and December followed by 3.68 mm/day during April. This is because April and November months are rainy months, while December also receives more at 50 mm of rainfall. The monthly average value of ETo was found to be 4.27 mm/day.

Soil texture of the site

Based on the soil analyses, the soil texture classes of the experimental field under this study, is made up of sandy loam at the top layer and clay loam at 40 to 60 cm. It was also found, that from 0 to 20 cm depth, clay content was 19%. From 20 to 40 cm depth, clay content was increased to 30 and 38% for the depth of 60 cm. This indicated that clay content increased as the depth increased. The important property of the soil of the Cyabayaga wetland for use as a growing paddy is due to the high moisture holding capacity of clay as presented in Table 1.

Soil bulk density of the area

Soil samples were taken from three selected sites and dried in the laboratory for further analysis. Soil mass was measured using an electronic balance; and its volume

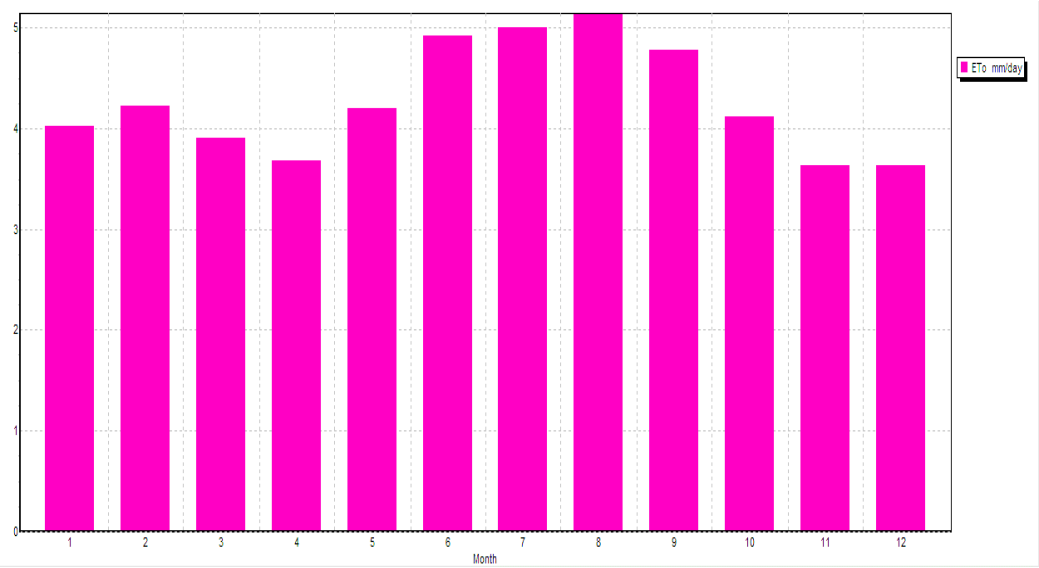

Figure 3. Reference evapotranspiration (ETo) for the site.

was calculated by measuring the volume of a core cutter having 5 cm of height and 5 cm of diameter:

$\mathbf{v = \pi r^2 h}$= 98.2 cm^3

The average bulk density of three plots was found to be 1.3 g/cm^3. It is evident that the soil of experimental site is within the range of bulk density that is defined to give ideal root growth.

Infiltration rate of soil for the experimental area

The infiltration rate for the site was determined by using a double ring infiltrometer. As defined by Yewle et al. (2016), basic infiltration rate found in study area falls in the category of moderately slow and the average infiltration rate of the site is 1.0 cm/h, which also falls under moderately slow infiltration rate.

Hydraulic conductivity of soil for the site

The measure of how water can pass easily within the soil is called hydraulic conductivity. Higher values indicate that the soil is highly permeable, while the lower values indicate less permeable soil. It is evident that the average hydraulic conductivity of the experimental plots selected in the study area are 0.0034 cm/s, 0.0038 cm/s and 0.0036 cm/s for Plots 1, 2, and 3, respectively. According to Humberto et al. (2002) and Blanco-Canqui et al. (2002), the hydraulic conductivity values of the soils of Cyabayaga wetland are rapid permeable. To reduce the deep percolation losses, farmers perform puddling operations. The latter refer to the tillage of rice paddies while flooded, an old practice that is practiced to prepare for rice cultivation by reducing the percolation rates of water through churning the clay soil particles and making them close many of the soil pores. Over time, soil puddling also creates a compacted layer under the puddled zone which further minimizes percolation loss of irrigation water.

Computation of irrigation requirements

Net irrigation requirement (NIR) refers to the artificial application of water to the field such that fulfill plants can use it for full plant growth without considering any water losses; while crop water requirement (CWR) is defined as the water used by the plant until it reaches maturity including water losses by seepage, land preparation, transplanting and evapotranspiration. Rice crop was planted on 23rd of July and harvested after 180 days on the 18th of January. Crop water requirement (ETcrop) and irrigation requirements (IR) have been computed on a decade and daily basis. The results are presented in Figure 4.

Referring to the Figure 4, it is evident that the highest irrigation requirement started from July to August because these months fall into long dry season and the irrigation requirement reduced due to onset of short rainy season from mid-September to mid-December. The lowest irrigation requirement was found to be November at 16.4 mm. The irrigation requirement was from Mid-December to Mid-January; which is relatively low, because the crop is at maturity. The total irrigation water requirement for the rice crop was 530.2 mm.

Table 1. Textural classes for exponential sites.

Depth	Soil particles %	Experimental plots taken from the study area			Average
		Plot 1	Plot2	Plot 3	
0-20 cm	Sand	50	71	62	61
	Silt	27	14	20	20
	Clay	23	15	18	19
	Textural class based on textural triangle	Sand clay loam	Sandy Loam	Sandy loam	Sandy loam
20-40 cm	Sand	60	31	31	41
	Silt	15	35	37	29
	Clay	25	34	30	30
	Textural class based on textural triangle	Sand Clay Loam	Clay Loam	Clay Loam	Clay Loam
40-60 cm	Sand	25	24	50	33
	Silt	40	26	22	29
	Clay	35	50	28	38
	Textural class based on textural triangle	Clay Loam	Clay	Sand Clay loam	Clay loam

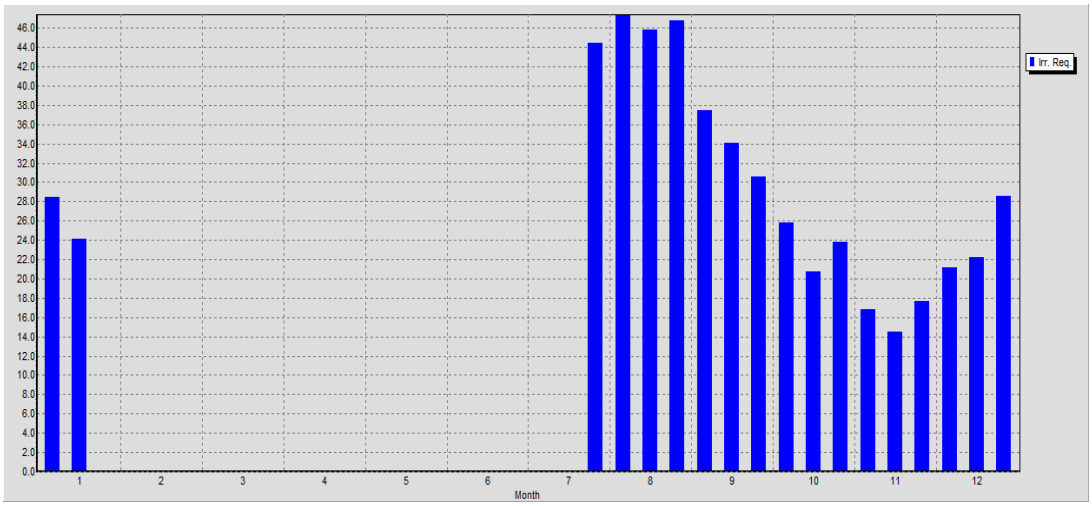

Figure 4. Irrigation requirement for Cyabayaga wetland.

Conveyance efficiency of irrigation channel in the study area

The conveyance efficiency represents the efficiency of water transport in the channel. The water conveyance efficiency is the ratio of water conveyed in one location to another location of the channel. The present study was conducted in earthen or unlined channel of the primary, secondary and tertiary channel levels. Water flow in three successive places of the channel was measured so as to find out the conveyance efficiency between two successive places.

Table 2 shows that, the conveyance efficiency in primary, secondary, and tertiary channels are 70.91, 73.91 and 60%, respectively. The overall average conveyance efficiency (Ec) = 70.05%

From the results, about 30% of water is lost through seepage and evapo-transpiration by undesired vegetation and leakage through water control structures in the unlined and poorly maintained conveyance system. In the study area also, it was found that the canals are not well maintained.

Daily water losses in the study area

The average water loss in each plot was measured at an interval of 24 h for 2 days and the mean value of water

Table 2. Conveyance efficiency in irrigation channels for the site.

Type of channel	Discharge of 3 places of the channels (m^3/s)			Conveyance efficiency between two consecutive places in the channel (%)		Average
	Place1	Place 2	Place 3	Between place 1 and 2	Between place 2 and 3	
Primary	0.74	0.55	0.39	74.32	70.91	72.62
Secondary	0.33	0.23	0.17	69.70	73.91	71.81
Tertiary	0.07	0.05	0.03	71.43	60.00	65.71
Average Ec (%)				-		**70.05**

losses for the three selected sites and 9 plots was computed. The results of water losses in irrigated plots are presented in Table 3. The average water losses in all selected sites is = $\frac{24+27+24}{3}$ = 25 mm.

From Table 3, the average water losses in the three selected sites of marshlands were found to be 24, 27 and 24 mm, respectively. It was also found that the losses in the first 24 h were less than the next 24 h. This may be due to more water required to fill the unsaturated pores. The average of all sites was determined to be 25 mm. The average water loss, that includes ET_c, deep percolation and seepage, is 25 mm/day.

The average water losses including ET_c and deep percolation and seepage are 25 mm/day. Considering that irrigation would be stopped before 2 weeks of harvesting, the amount of water losses have been considered for 166 days. Based on ETc for 166 days the average ETc losses of 4.8 mm was taken as the actual water loss calculations, hence, 4.8 mm/day is taken as the water depletion from the rooting zone, then the extra water which was not used by the crop was 25-4.8 = 20.2 mm. Hence, losses due to deep percolation and seepage are 20.2 mm/day.

Furthermore, water application efficiency Ea = 4.8 × 100/20.2= 23.76%, or about 23.8%. The overall efficiency would be Ec × Ea = 0.70 × 0.238 × 100 = 16.67% or approximately 16.7%. Considering net irrigation of 584.6 mm given by Cropwat 8.0 with 70% irrigation efficiency, this results to 835.4 mm. In actual field conditions based on measurements made during the present study, the actual irrigation efficiency was found to be 16.67%. This indicates that a huge amount of irrigation water is lost in field conditions and caused by seepage and percolation. Actually, the farmers of Cyabayaga marshland are currently using 4771.3 mm when crop evapotranspiration needs are 796.8 mm.

It means that the total water of 4771.3 m^3 is being used for each hectare of land and rice yields (4.25 tons/ha) are also low due to poor input management.

Water use efficiency

The water ratio of crop yield (Y) to the total amount of water used (WR) in the field is called water use efficiency (WUE). WUE = Y/WR

The average rice production of 4.25 tons/ha (Miklyaev et al., 2021) was taken similar to production of Nyagatare district where Cyabayaga wetland is located. It is clear that 4771.3 m^3 of water is required to produce 4.25 tons of rice from 1 ha. This means that to produce 1 kg of rice a volume of 11.23 m^3 of water is expected to be required. Hence, the water use efficiency would be 1/11.93 m^3= 0.089 kg/m^3. It shows a poor water use efficiency and there is a need to improve it.

Research conducted by Jaffar and Sitha (2017) revealed that the production and efficiency of water use for rice is comparable to irrigation scheduling in transplanted rice gave 0.36 kg/m^3 WUE. It also indicates the importance of irrigation scheduling for efficient use of water.

In the study area, farmers are flooding 10 to 20 cm of water that also makes a huge amount of water loss in application. To control such water losses, proper land leveling, puddling and appropriate irrigation scheduling improve water management and higher rice yields. Therefore, farmers have to manage smaller amounts of water in their fields.

Conclusion

The data collected, analyzed and discussed in this paper brought the following points as conclusions of this research. The highest average minimum temperature is 15.6°C, which was found in April and average lowest minimum temperature was 13.9°C, found in June and July. The highest maximum temperature was 28°C found during August and September; and the lowest maximum temperature of 27°C was found in the months of July and November. It shows that in the study area, the temperature variations are quite low. The high relative humidity, ranging from 77 to 82%, were found during March, April, October, November, and December due to rainy seasons. The lowest relative humidity of 46% was found in the month of July, followed by August and June with 48 and 51%, respectively. These months fall under the long dry season. The highest rainfall of 107.9 mm was in the month of April. The months that occur during

Table 3. Daily field water loss for the study area.

Plot	Days of field experiment	Initial water level after irrigation (mm)	Water level after 24 h	water loss after 24 h (mm/day)	Water level after48 h	Water loss in next 24 h	Average water loss during 24 h
Site 1	1	150	122	28	96	26	27
	2	156	129	27	106	23	25
	3	145	123	22	105	18	20
Average water loss							24
Site 2	1	200	162	38	120	34	36
	2	145	120	25	99	21	23
	3	172	150	22	130	20	21
Average water loss				22			27
Site 3	1	160	136	24	116	20	22
	2	165	139	26	117	22	24
	3	148	120	28	96	24	26
Average water loss							24

the cropping season within the study area were July to January. During this period, the month of November had the highest rainfall of 98.9 mm, followed by 97.8, 71.8, 64.1, and 25.7 mm during the cropping season. From the rainfall data and irrigation scheduling, it can be concluded that less irrigation was required during the rainy months and was more when amount of monthly rain was less. It was also found that the soils of experimental sites are comprised sandy loam at the top layer with clay loam beneath. The average bulk density of the Cyabayaga wetland was found to be 1.3 g/cm^3, and it defines the ideal root growth. The basic infiltration rate was found to be 1.0 cm/h, and the infiltration rate of the area falls under moderately slow infiltration rate. The average hydraulic conductivity of soils in the study area are highly permeable with 12.96 cm/h (rapid category) leading to enormous water losses. The results indicated that the highest ETc of 5.6 mm/day was found in the month of September and lowest ETc of 3.72 mm was during January.

The total irrigation requirement of 530.2 mm for the entire season was given from Cropwat 8.0; where an ideal irrigation efficiency of 70% was kept, which is not the case of rice irrigation all over the world. Therefore, actual losses in the field were measured. The conveyance efficiency (Ec) is found to be 70.05%, showing 30% of water lost through seepage and evapotranspiration through weeds in unlined channels. The overall deep percolation, seepage and evapotranspiration losses from flooded rice fields were found to be the range of 25 mm/day. Considering the highest ETc of 4.8 mm, it was concluded that a huge water loss of 20.2 mm/day was caused by seepage and percolation. The application efficiency (Ea) of 23.8% was found in the study area; hence, the overall efficiency was calculated to be 16.7%. With the current rice yield of 4.25 t/ha, a low water use efficiency of 0.089 kg/m^3 was found. Therefore, an integrated approach of land and water management is highly needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Spatial and regional directory of wild *Auricularia* species found in the forest of Southwest Nigeria

Victor S. Ekun[1], Clementina O. Adenipekun[1], Omena B. Ojuederie[2] and Peter M. Etaware[1*]

[1]Department of Botany, Faculty of Science, University of Ibadan, Oyo State, Nigeria.
[2]Food Security and Safety Niche Area, Faculty of Natural and Agricultural Sciences, North-West University, Private Bag X2046, Mmabatho, 2735, South Africa.

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The African forest, which was and still is the heart of biodiversity, is fast losing its bio-components due to natural disasters and human encroachment. A catalogue of the bio-components of the African forest is imminent so that conservation programs can be established to safeguard rare germplasm(s) from extinction. The population density of wild *Auricularia* (Mushroom) of Southwestern Nigeria was the focus of this research. DNA primers were obtained from Operon Technology, Alameda, California, USA. Characterization was done using phenotype, PCR and electrophoresis gel (RAPD). OPB-11 to OPB-21, OPH-3 to OPH-15, and OPT-1 to OPT-19 primers formed polymorphic bands with DNA samples of the specimens. Three (3) distinct species of *Auricularia* were identified in the forest of Southwest Nigeria based on PCR and RAPD analyses. Geospatial analysis showed that *Auricularia auricula* was present in the forests of Ekiti, Osun, Ogun, Oyo and Lagos states, but none was identified in Ondo State. On the contrary, *Auricularia polytricha* was only identified in the tropical rainforest and grassland vegetation of Ondo, Lagos and Oyo states.

Key words: *Auricularia*, mushrooms, population density, Southwestern Nigeria, phenotypic description, genome similarity index.

INTRODUCTION

The oldest documentary of mushroom scavenging by humans was discovered in Tassili (Southern Algeria), Acacus (Libya) and Ennedi (Chad), etc., where the historical data was well preserved in magnificent rock drawings, paintings and other artifacts of the earliest inhabitants of Africa (also known as the cradle of humanity). So far, the mycoflora of modern Africa is still largely unknown (Gram, 2021). Mushroom foraging was and still remain a promising means of generating income within rural settlements in Africa, since it is a highly-sought-after nutritional (food) supplement, and a pertinent raw material for folk medicine (Degreef et al., 2016). In ancient times, the African woodland terrain was teeming with different variety of micro- and macroflora capable of biodegradation, such that the problem of waste management never arose since these “natural recyclers” efficiently and effortlessly transformed the huge amount of wastes deposited indiscriminately, on daily basis, in the environment (Degreef et al., 2016; Etaware, 2021). These florae (basically macro-fungi) were capable of

*Corresponding author. E-mail: peterparkers007@gmail.com or peter.etaware@nmu.edu.ng.

degrading recalcitrant wastes and they even had the propensity to absorb and store toxic elements from the environment (Adenipekun et al., 2015; Etaware, 2021).

The macro-fungi (mushroom) population of the African forest is fast declining and they may become endangered in the nearest future due to extermination of their physical domain by climate change, natural disaster, animal and human intervention (Osemwegie et al., 2014). A direct catalogue and geo-referencing of the existing mushroom species within the African forest is imminent so that anti-grazing or anti-poaching laws refraining human activities in the coveted areas can be promulgated and other conservation programs can be established to safeguard the existing mushroom germplasms and prevent them from going extinct. *Auricualaria* species are highly priced mushrooms with nutritional and medicinal benefits (Chang and Hayes, 2013), they are currently ranked third in global mushroom production behind *Lentinula* and *Pleurotus* species (Royse et al., 2017; Bandara et al., 2019). In terms of distribution, *Auricularia* spp. are cosmopolitan, sadly, the species diversity within the genus *Auricularia* is on the decline. Currently, there are only less than 10 identifiable *Auricularia* spp. in China and a little below 25 species existing around the world (Wu et al., 2015).

In the rural and sub-urban areas of Africa, the most reliable means of identification of edible mushrooms by the indigens are mostly through phenotypic (physical) appearance, based on innate experience acquired through long term mushroom foraging in the wild. This is very risky, as the difference between a poisonous and edible mushroom is not always defined by physical appearance. Also, some researchers have been able to classify mushrooms based on physical appearance alone (Onyango et al., 2011). The use of physical appearance in the identification of edible mushrooms (*Auricularia* spp. inclusive) in some parts of Africa (especially Nigeria) is quite risky, unscientific, unreliable, misleading and may be ineffective on the long-run, since phenotypic classifications can be greatly influenced or affected by slight variation of environmental factors. Therefore, the current research was setup to identify the existing population of *Auricularia* spp. within the tropical forest of Southwestern Nigeria using sophisticated molecular techniques (PCR and RAPD), with a view to ensure proper documentation of the available species of *Auricularia* in the wild and their geographical location. Part of the aim of this research was to ensure that the mushroom germplasms collected for conservation were properly identified (Moore, 2013).

Taxonomy of Auricularia

Kingdom: Fungi
Division: Eumycota (Basidiomycota)
Sub-division: Basidiomycotina
Class: Agaricomycetes
Order: Auriculariales
Family: Auriculariaceae
Genus: Auricularia

MATERIALS AND METHODS

Research area

The tropical rainforest, mangrove swamp and savannah vegetation of the Southwestern region of Nigeria (Figure 1) was selected as the case study of this research. The zone is characterized by a bi-seasonal weather condition (Rainy and dry season), with sufficient annual precipitation (rainfall), ambient sunshine, high relative humidity and a warm day time temperature. Southwest Nigeria is one of the six (6) geo-political zones of the country, located on Latitude 9.081999° N and Longitude 8.675277°E, respectively. It is made up of six (6) states, that is, Ekiti, Lagos, Ogun, Ondo, Osun and Oyo states as shown in Figure 2.

The proposed sites for *Auricularia* catalogue in Southwest Nigeria

The proposed locations for mushroom foraging in Southwestern Nigeria were structured from the description given by renowned indigenous mushroom collectors. Sample stations were drawn from the six (6) states of Southwest Nigeria, covering all the possible terrain and forest locations within the earmarked area where mushrooms can thrive (Table 1). The forest and savannah (grassland) region of Southwest Nigeria was thoroughly surveyed towards the end of the rainy season in 2011 to the peak of the wet season in 2012. The features pertinent for effective identification of wild *Auricularia* spp. were adapted from the research of Musngi et al. (2004). The pattern of investigation of the wild forest of Southwest Nigeria is as shown in Figures 1 to 3.

Phenotypic identification of wild *Auricularia*

The identification of *Auricularia* spp. was done using both the traditional method (phenotypic characters only) and molecular characterization (using genome similarity). The traditional method was described in this session. A quick in-field identification was done by the researchers/collectors using the phenological description given by Onyango et al. (2011). The pre-identified *Auricularia* samples collected from the earmarked location within the wild forest of the six (6) states of Southwest geopolitical zone of Nigeria were aseptically packaged in sterile propylene bags and labelled appropriately (that is, sample site, coordinates, time of collection, and date). The collected samples were thereafter transferred to the mushroom house of the Department of Botany, University of Ibadan, Ibadan, Oyo State for further identification. Mushroom samples were sorted initially based phenotypic (morphological) characters with special focus on the appearance of the fruiting body and the presence or absence of reproductive structures (hymenium). The method adapted was a simplistic protocol used by native Africans in the identification of edible mushrooms (*Auricularia* spp. Inclusive), which was described by Onyango et al. (2011). The phenotypic characters investigated are shown in Table 2. Furthermore, anatomical studies of the collected samples were carried out in the laboratory, both at the University of Ibadan and National Institute of Horticulture (NIHORT), Ibadan, Oyo State, Nigeria. The spores from the basidiocarps of each mushroom was inoculated into full-strength PDA (in replicates of 3) and cultured for 7 days at 25±2°C, the mycelial morphology of the spawn produced were noted too.

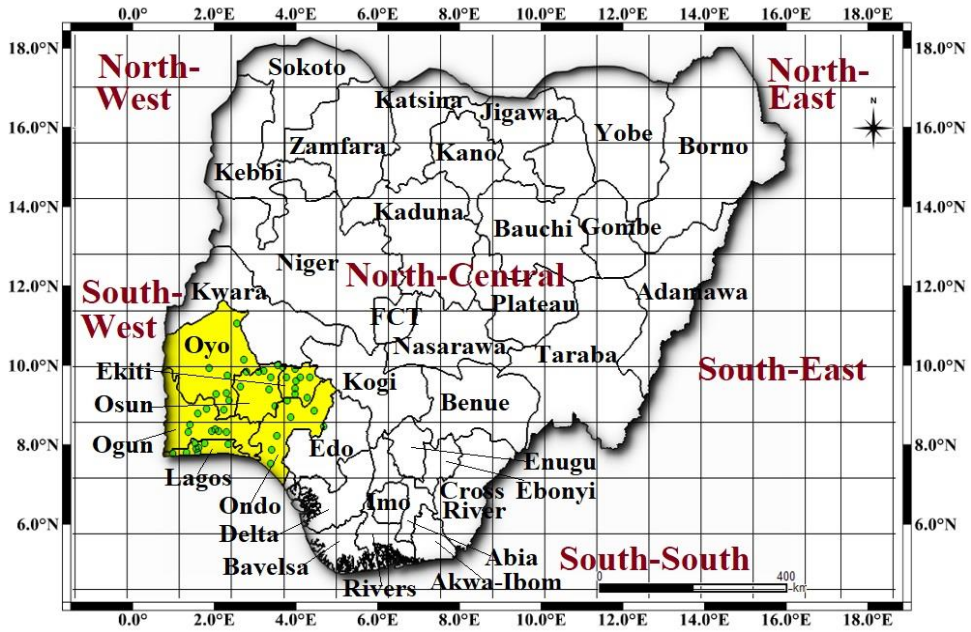

Figure 1. An overview of the Nigerian terrain.

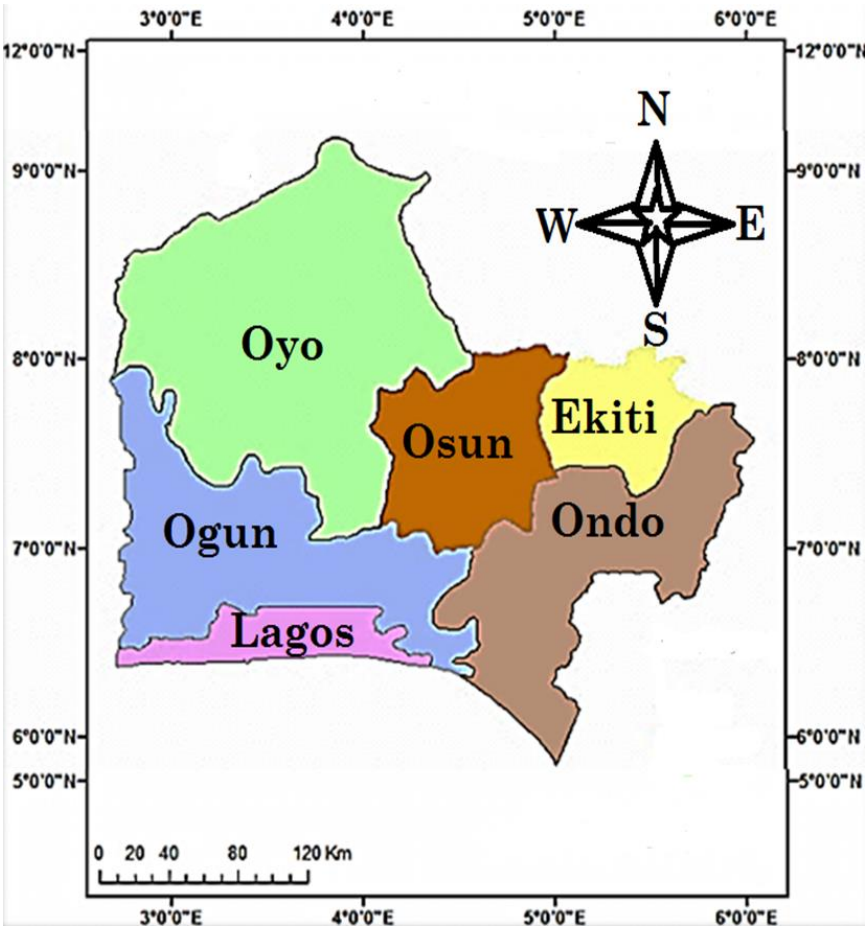

Figure 2. The map of Southwestern Nigeria showing the conglomerated states.

Table 1. The proposed locations for mushroom foraging in Southwestern Nigeria.

S/N	Local Govt. Area	Location	Province
1	Abeokuta North	Abeokuta	Ogun
2	Ewekoro	Itori	Ogun
3	Ifo	Ifo	Ogun
4	Ijebu Ode	Ijebu Ode	Ogun
5	Ikenne	Ikenne	Ogun
6	Shagamu	Shagamu	Ogun
7	Odeda	Odeda	Ogun
8	Odogbolu	Odogbolu	Ogun
9	Agege	Ikeja	Lagos
10	Ojo	Ojo	Lagos
11	Apapa	Ikeja	Lagos
12	Badagry	Badagry	Lagos
13	Epe	Epe	Lagos
14	Shomolu	Shomolu	Lagos
15	Ikorodu	Ikorodu	Lagos
16	Mushin	Ikeja	Lagos
17	Akinyele	Moniya	Oyo
18	Egbeda	Egbeda	Oyo
19	Ido	Ido	Oyo
20	Iseyin	Iseyin	Oyo
21	Ogbomosho North	Ogbomosho	Oyo
22	Oluyole	Idi Ayunre	Oyo
23	Oyo	Oyo	Oyo
24	Olorunsogo	Igbeti	Oyo
25	Akinyele	Ojo	Oyo
26	Ibadan North	Bodija	Oyo
27	Ado Ekiti	Ado Ekiti	Ekiti
28	Ilejemeje	Iye	Ekiti
29	Ikole	Ikole	Ekiti
30	Oye	Oye	Ekiti
31	Irepodun	Igede	Ekiti
32	Ikere	Ikere	Ekiti
33	Ijero	Ijero Ekiti	Ekiti
34	Emure	Emure Ekiti	Ekiti
35	Idanre	Idanre	Ondo
36	Ilaje	Igbokoda	Ondo
37	Ile Oluji	Ile Oluji	Ondo
38	Odigbo	Ore	Ondo
39	Okitipupa	Okitipupa	Ondo
40	Ose	Ifon	Ondo
41	Owo	Owo	Ondo
42	Ifedore	Igbara Oke	Ondo
43	Akure South	Akure	Ondo
44	Bolunduro	Ota Aiyebaju	Osun
45	Ejigbo	Ejigbo	Osun
46	Ifedayo	Oke Ila Orangun	Osun
47	Ifelodun	Ikirun	Osun
48	Ila	Ila Orangun	Osun
49	Irepodun	Ilobu	Osun
50	Iwo	Iwo	Osun
51	Obokun	Ibokun	Osun

Table 1. Contd.

52	Irewole	Ikire	Osun
53	Oriade	Ilesha	Osun
54	Oriade	Ipetu Ijesha	Osun

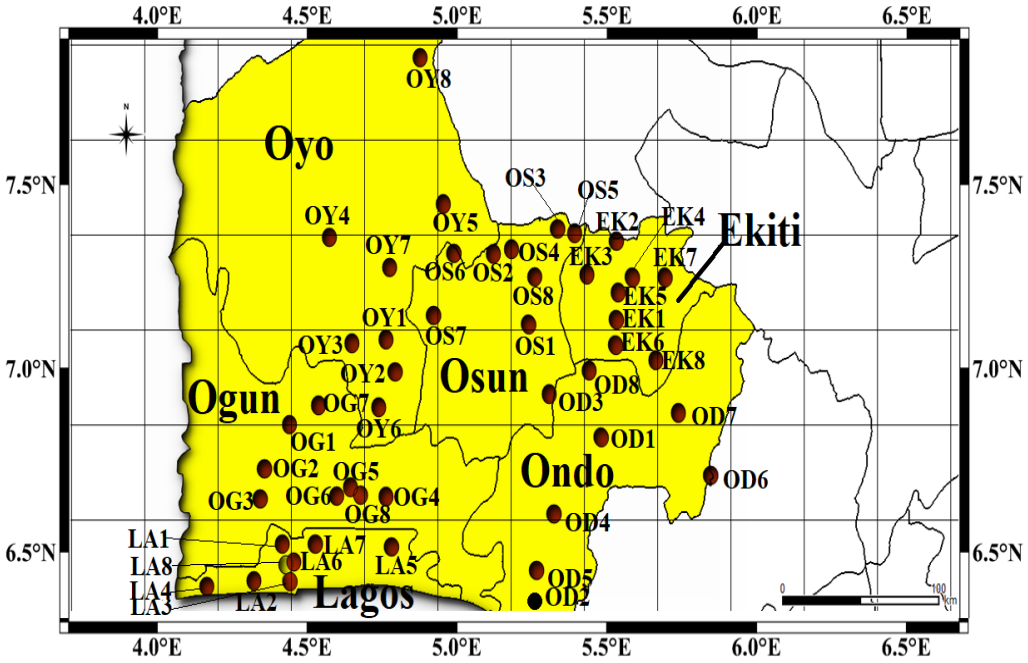

Figure 3. The map of Southwestern Nigeria showing the sample areas for *Auricularia* mushrooms.

Table 2. Phenotypic classification of *Auricularia* samples collected from the wild.

S/N	External features					Inference	
	Fruiting Body			Mycelia		Identity	
	Shade	Outline	Appearance	Shade	Appearance	General	Specific
1	Dusky chocolate colour	Circular	Jellylike	White	Cotton-like	*Auricularia*	*Auricularia* spp.
2	Creamy chocolate colour	Ear-shaped	Fibrous	Grey	Cotton-like	"	*A. auricula*
3	Tan or chocolate colour	Flat	Flexible	Grey	Scanty growth	"	*Auricularia* spp.
4	Dusky chocolate colour	Circular	Jellylike	White	Cotton-like	"	*A. polytricha*

Phenotypic description of *Auricularia* spp.
Source: Onyango et al. (2011).

Genome classification of the collected mushroom samples

Extraction and purification of mushroom DNA

Standard protocol for extraction of DNA from mushroom samples described by Chen et al. (2010) was adopted for this research. The steps involved are as follows:

(1) Aseptically excise about 200 mg of rehydrated tissue from the pileus of each mushroom sample
(2) Place the fragments of the excised tissue in an electric grinder with about 800 mL of CTAB buffer and pulverize to form a paste
(3) Incubate the pulverized samples at 65°C for 15 min in a water bath with constant agitation to allow even mixture
(4) Allow the mixture to cool before adding a composite solution of phenol, chloroform and iso-amyl in a ratio of 25:24:1
(5) Centrifuge the resulting mixture at 12,000 rpm for 15 min using a standard laboratory centrifuge
(6) Aseptically dispense the supernatant into sterile test tubes before adding about 400 µL of isopropanol (stored at 4°C).

Table 3. Primers used in this research for genomic study.

S/N	Primer code	Primer sequence	Melting Point (Tm^{o}C)
1	OPB-11	5^{l} GTAGACCCGT 3^{l}	34
2	OPB-12	5^{l} CGTTGACGCA 3^{l}	34
3	OPB-15	5^{l} GGAGGGTGTT 3^{l}	32
4	OPB-20	5^{l} GGACCCTTAC 3^{l}	34
5	OPB-21	5^{l} CGACCCTTAC 3^{l}	34
6	OPH-3	5^{l} AGACGTCCAC 3^{l}	34
7	OPH-5	5^{l} AGTCGTCCCC 3^{l}	32
8	OPH-10	5^{l} CCTACGTCAG 3^{l}	32
9	OPH-15	5^{l} GCTTCGTCAG 3^{l}	34
10	OPT-1	5^{l} GGGCCACTCA 3^{l}	34
11	OPT-5	5^{l} GGGTTTGGCA 3^{l}	32
12	OPT-7	5^{l} GGCAGGCTGT 3^{l}	34
13	OPT-10	5^{l} CCTTCGGAAG 3^{l}	32
14	OPT-19	5^{l} GATGCCAGAC 3^{l}	32

Operon Technology, Alameda, California, USA.

(7) Homogenize the mixture by flipping manually up to five times to aid effective precipitation of the DNA
(8) Store at -80°C for 1 h.
(9) Centrifuge again at 12,000 rpm for 10 min to obtain DNA pellets.
(10) Decant the initial solvent from the pelleted DNA samples and rehydrate with 100 µL sterile distilled water and 2 µL of 10 mg/mL RNase
(11) Store at 4°C for 30 min (at this point, fragments of RNA strands will be dissolved completely).

DNA/RNA primer procurement

The primers used for this research were obtained from Operon Technology (Alameda, CA, USA) (Table 3).

Auricularia spp. genome profiling using PCR and RAPD

The genome identity of the pre-identified *Auricularia* samples collected in the wild was determined using PCR and profiled on electrophoresis gel (RAPD). The steps involved were described as the following.

PCR analysis

Reagents: A = Buffer (10x), B = 50 mM $MgCl_2$, C = 2.5 mM dNTPs,

D = 500 U *Taq* DNA polymerase, E = Dimethyl sulfoxide (DMSO), and F = DEPC-treated water.

Procedure:

(1) An aliquot of 2.0 µL of the standardized DNA fragments (100 ng) was loaded into the tube of an Applied Bio-system thermo-cycler
(2) A mixture of 25 µL of A, 1.25 µL of B, 2.0 µL of C, and 0.2 µL of D were added to the loaded DNA fragment in the tube
(3) Furthermore, 1.0 µL of E, 1.0 µL of each primer and 16.05 µL of F were added to the mixture in the tube too.
(4) PCR amplification were conducted at 40-60 cycles at 94^{o}C for 2 mins (initial cycling speed), 40 cycles at 94^{o}C for 20 s, then at 72^{o}C for 1 min., and at 54^{o}C for 2 mins. Finally, run the sample at a speed of 40 cycles for 5mins at 72^{o}C
RAPD analysis
(5) An aliquot of about 2.5 µL of the already extracted and purified DNA sample obtained from the pre-identified *Auricularia* specimen was aseptically loaded into the compartments or wells of the RAPD device containing 1.5% agarose gel, for electrophoresis gel analysis.
(6) The loaded sample was observed under ultraviolet radiation (UV) to ascertain the quality of the samples extracted.
(7) The DNA fragments were separated based on their electron affinity and size
(8) Afterwards, sample uniformity was ensured by calibrating the separated DNA fragments to a value of 100 ng/µL.

Quantification of the calibrated and purified DNA samples

The Nano-Drop spectrophotometer (recommended) was used to quantify the already calibrated DNA samples obtained via the electrophoresis gel analysis. The criteria for proper quantification were stated thus:

(1) Sample size: 2 µL
(2) Optimum ratio (R): 1.8:2.0
(3) Optimum absorbance level/Optical density (OD): 260/280 nm

$$\text{Absorbance level/OD} = \text{Log}_{10}\left(\frac{X_{cd}}{Y_{cd}}\right)$$

where X_{cd} = the intensity of the incident light and Y_{cd} = the intensity of the transmitted light.

Statistical analysis

The data obtained from the electrophoresis gel analysis were scored as present (1) or absent (0). The dissimilarity matrix was ascertained by value of the Jaccard's similarity coefficient (using the Jaccard Standard Protocol). Phylogenetic relations were determined by cluster analysis using UGPMA aided by the NTSYS-pc software

Table 4. Geo-mapping of *Auricularia* species in Ogun, Nigeria based on morphological characters.

Tag		Location		GPS Coordinate		Status	
Station	Code	Local govt. area	Town	Latitude	Longitude	Genus	Species
01	OG1	Abeokuta North	Abeokuta	7.1475°N	3.3619°E	*Auricularia*	Unknown
02	OG2	Ewekoro	Itori	6.9530°N	3.2181°E	"	Unknown
03	OG3	Ifo	Ifo	6.8192°N	3.1930°E	"	*auricula*
04	OG4	Ijebu Ode	Ijebu Ode	6.8300°N	3.9165°E	"	*auricula*
05	OG5	Ikenne	Ikenne	6.8717°N	3.7105°E	"	*auricula*
06	OG6	Shagamu	Shagamu	6.8322°N	3.6319°E	"	*auricula*
07	OG7	Odeda	Odeda	7.2328°N	3.5281°E	"	*auricula*
08	OG8	Odogbolu	Odogbolu	6.8365°N	3.7689°E	"	*auricula*

OG: Ogun.

version 2.02. Phylogenetic characterization into multivariate groups was done using principal component analysis (PCA) with Darwin software version 5.0.0.157 while polymorphic information content (PIC) was calculated using the method described by Botstein and colleagues in 1980.

RESULTS

Auricularia spp. stereotyping and mapping in Nigeria

Auricularia spp. were identified in forty-eight (48) out of the fifty-four (54) locations initially earmarked for this research (Figure 3). A total of eight (8) locations were geo-referenced in each state where these edible mushrooms species can be found. It was noted that *Auricularia* spp. were abundantly present in the forest and grassland region of Abeokuta, Itori, Ifo, Ijebu Ode, Ikenne, Shagamu, Odeda, and Odogbolu in Ogun State (Table 4), Agege, Ojo, Apapa, Badagry, Epe, Shomolu, Ikorodu, and Mushin in Lagos State (Table 5), Moniya, Egbeda, Ido, Iseyin, Ogbomosho, Idi Ayunre, Oyo, and Igbeti in Oyo State (Table 6), Ado Ekiti, Iye, Ikole, Oye, Igede, Ikere, Ijero Ekiti, and Emure Ekiti in Ekiti State (Table 7), Idanre, Igbokoda, Ile Oluji, Ore, Okitipupa, Ifon, Owo, and Igbara Oke in Ondo State (Table 8), and finally, it was also sighted in places like Bolunduro, Ejigbo, Ifedayo, Ifelodu, Ila, Irepodun, Iwo, and Obokun in Osun State (Table 9).

The species *A. auricula* was identified in 6 communities in Ogun State (Ifo, Ijebu Ode, Ikenne, Shagamu, Odeda, and Odogbolu) (Table 4), 4 communities in Lagos (Agege, Ojo, Apapa, and Badagry) (Table 5), 5 communities in Oyo State (Egbeda, Ogbomosho, Idi Ayunre, Oyo, and Igbeti) (Table 6), 8 communities in Ekiti and Osun States (Ado Ekiti, Iye, Ikole, Oye, Igede, Ikere, Ijero Ekiti, Emure Ekiti) (Table 7), Bolunduro, Ejigbo, Ifedayo, Ifelodu, Ila, Irepodun, Iwo, and Obokun (Table 9), respectively. None was recorded for Ondo State (Table 8). On the contrary, *A. polytricha* was found in abundance in all the locations in Ondo State, that is, Idanre, Igbokoda, Ile Oluji, Ore, Okitipupa, Ifon, Owo, and Igbara Oke (Table 8), 3 locations in Lagos State (Shomolu, Ikorodu and Mushin) (Table 5), and one (1) location in Oyo State that is, Moniya (Table 6). The research team was unable to identify a rare *Auricularia* spp. in some part of the research area (Tables 4 to 6), further genomic analyses are required to effectively identify this rare species.

Genome classification of the collected mushroom samples

Quality assessment of the extracted Auricularia DNA

Majority of the DNA samples extracted for use in this experiment were pure ($A_{260/280} \geq 1.8$), that is, high-quality DNA extracts, with the exception of OG2 ($A_{260/280} = 1.78$), OG6 ($A_{260/280} = 1.76$) (Table 10), EK2 ($A_{260/280} = 1.74$), EK3 ($A_{260/280} = 1.75$) (Table 13), OD2 ($A_{260/280} = 1.67$), OD8 ($A_{260/280} = 1.62$) (Table 14), and OS4 ($A_{260/280} = 1.75$) (Table 15), with little protein and RNA contaminants found in their DNA extracts. All the DNA samples extracted from the prospective *Auricularia* specimen collected from the tropical forest in Lagos and Oyo states were pure, with minimal impurities, that is, $A_{260/280} \geq 1.8$ (Tables 11 and 12). It was observed that *Auricularia* specimen collected from Station 8 in Ogun State had the highest quantity of pelleted DNA sample with 1,548.2ng/μL of pure concentrated nucleic acid (Table 10). The least quantity of DNA extracts was obtained from *Auricularia* mushroom samples within Station 6 in Ondo State (56.5ng/μL of Nucleic acid concentration) (Table 14).

Genetic variability within the genus "Auricularia"

The major allele frquency of distribution of DNA fragments was highest on the primer location "OPH-15" (60%) and it was least on the primer location "OPB-15"

Table 5. Geo-mapping of *Auricularia* species in Lagos, Nigeria based on morphological characters.

Tag		Location		GPS Coordinate		Status	
Station	**Code**	**Local govt. area**	**Town**	**Latitude**	**Longitude**	**Genus**	**Species**
09	LA1	Agege	Ikeja	6.6180°N	3.3209°E	*Auricularia*	*auricula*
10	LA2	Ojo	Ojo	6.4579°N	3.1580°E	"	*auricula*
11	LA3	Apapa	Ikeja	6.4553°N	3.3641°E	"	*auricula*
12	LA4	Badagry	Badagry	6.4316°N	2.8876°E	"	*auricula*
13	LA5	Epe	Epe	6.6055°N	3.9470°E	"	Unknown
14	LA6	Shomolu	Shomolu	6.5392°N	3.3842°E	"	*polytricha*
15	LA7	Ikorodu	Ikorodu	6.6194°N	3.5105°E	"	*polytricha*
16	LA8	Mushin	Ikeja	6.5273°N	3.3414°E	"	*polytricha*

LA: Lagos.

Table 6. Geo-mapping of *Auricularia* species in Oyo, Nigeria based on morphological characters.

Tag		Location		GPS Coordinate		Status	
Station	**Code**	**Local govt. area**	**Town**	**Latitude**	**Longitude**	**Genus**	**Species**
17	OY1	Akinyele	Moniya	7.5249°N	3.9152°E	*Auricularia*	*polytricha*
18	OY2	Egbeda	Egbeda	7.3796°N	3.9675°E	"	*auricula*
19	OY3	Ido	Ido	7.5077°N	3.7194°E	"	Unknown
20	OY4	Iseyin	Iseyin	7.9765°N	3.5914°E	"	Unknown
21	OY5	Ogbomosho North	Ogbomosho	8.1227°N	4.2436°E	"	*auricula*
22	OY6	Oluyole	Idi Ayunre	7.2247°N	3.8732°E	"	*auricula*
23	OY7	Oyo	Oyo	7.8430°N	3.9368°E	"	*auricula*
24	OY8	Olorunsogo	Igbeti	8.7699°N	4.1104°E	"	*auricula*
52	OY9	Akinyele*	Ojo*	7.5503°N	3.9470°E	None	None
53	OY10	Ibadan North*	Bodija*	7.4351°N	3.9143°E	None	None

OY: Oyo; *: No *Auricularia* specimen found in the region.

Table 7. Geo-mapping of *Auricularia* species in Ekiti, Nigeria based on morphological characters.

Tag		Location		GPS coordinate		Status	
Station	**Code**	**Local govt. area**	**Town**	**Latitude**	**Longitude**	**Genus**	**Species**
25	EK1	Ado Ekiti	Ado Ekiti	7.6124°N	5.2371°E	*Auricularia*	*auricula*
26	EK2	Ilejemeje	Iye	7.9591°N	5.2371°E	"	*auricula*
27	EK3	Ikole	Ikole	7.7983°N	5.5145°E	"	*auricula*
28	EK4	Oye	Oye	7.7979°N	5.3286°E	"	*auricula*
29	EK5	Irepodun	Igede	7.7313°N	5.2476°E	"	*auricula*
30	EK6	Ikere	Ikere	7.4991°N	5.2319°E	"	*auricula*
31	EK7	Ijero	Ijero Ekiti	7.8120°N	5.0677°E	"	*auricula*
32	EK8	Emure	Emure Ekiti	7.4317°N	5.4621°E	"	*auricula*

EK: Ekiti.

(35%). The highest number of allele recorded during the genomic analysis was highest on the primer locations "OPB-21 and OPT-10" with a joint top score of 16. The least recorded number of allele was on primer loci "OPH-10 and OPH-15" with 5 allele each. The highest level of genetic diversity observed during the genomic analysis was on primer locus "OPB-12" with a potential genetic variability of 80%, while the least was on the primer location "OPH-15" with an average genetic variability of 59%. The highest level of DNA band polymorphism was

Table 8. Geo-mapping of *Auricularia* species in Ondo, Nigeria based on morphological characters.

Tag		Location		GPS coordinate		Status	
Station	**Code**	**Local govt. area**	**Town**	**Latitude**	**Longitude**	**Genus**	**Species**
33	OD1	Idanre	Idanre	7.0914°N	5.1484°E	*Auricularia*	*polytricha*
34	OD2	Ilaje	Igbokoda	6.2585°N	4.7692°E	"	*polytricha*
35	OD3	Ile Oluji	Ile Oluji	7.2825°N	4.8521°E	"	*polytricha*
36	OD4	Odigbo	Ore	6.7519°N	4.8780°E	"	*polytricha*
37	OD5	Okitipupa	Okitipupa	6.5025°N	4.7795°E	"	*polytricha*
38	OD6	Ose	Ifon	6.9235°N	5.7774°E	"	*polytricha*
39	OD7	Owo	Owo	7.1989°N	5.5932°E	"	*polytricha*
40	OD8	Ifedore	Igbara Oke	7.3877°N	5.0807°E	"	*polytricha*
54	OD9	Akure South*	Akure*	7.2571°N	5.2058°E	None	None

OD: Ondo; *: No *Auricularia* specimen found in the region.

Table 9. Geo-mapping of *Auricularia* species in Osun, Nigeria based on morphological characters.

Tag		Location		GPS coordinate		Status	
Station	**Code**	**Local govt. area**	**Town**	**Latitude**	**Longitude**	**Genus**	**Species**
41	OS1	Bolunduro	Ota Aiyebaju	7.5912°N	4.7329°E	*Auricularia*	*auricula*
42	OS2	Ejigbo	Ejigbo	7.9045°N	4.3052°E	"	*auricula*
43	OS3	Ifedayo	Oke Ila Orangun	7.9946°N	4.9974°E	"	*auricula*
44	OS4	Ifelodun	Ikirun	7.9227°N	4.6347°E	"	*auricula*
45	OS5	Ila	Ila Orangun	8.0121°N	4.8988°E	"	*auricula*
46	OS6	Irepodun	Ilobu	7.9021°N	4.5315°E	"	*auricula*
47	OS7	Iwo	Iwo	7.6292°N	4.1872°E	"	*auricula*
48	OS8	Obokun	Ibokun	7.8019°N	4.7692°E	"	*auricula*
49	OS9	Irewole*	Ikire*	7.3700°N	4.1872°E	None	None
50	OS10	Oriade*	Ilesha*	7.6395°N	4.7588°E	None	None
51	OS11	Oriade*	Ipetu Ijesha*	7.4273°N	4.9091°E	None	None

OS: Osun; *: No *Auricularia* specimen found in the region.

Table 10. Qualitative assessment of nucleic acid extracted from *Auricularia* species in Ogun State.

Station	Code	Town	Latitude	Longitude	Nucleic acid conc. (ng/µL)	$A_{260/280}$
01	OG1	Abeokuta	7.1475°N	3.3619°E	190.6	2.03
02	OG2	Itori	6.9530°N	3.2181°E	79.70	1.78
03	OG3	Ifo	6.8192°N	3.1930°E	250.0	1.87
04	OG4	Ijebu Ode	6.8300°N	3.9165°E	118.0	1.95
05	OG5	Ikenne	6.8717°N	3.7105°E	107.9	1.87
06	OG6	Shagamu	6.8322°N	3.6319°E	189.0	1.76
07	OG7	Odeda	7.2328°N	3.5281°E	92.10	2.02
08	OG8	Odogbolu	6.8365°N	3.7689°E	1548.2	1.89

OG: Ogun.

observed on primer locus "OPB-12" with 78% propensity for DNA band polymorphism. The least polymorphic information was noted at primer locus "OPH-15" with 56% chances of DNA polymorphism. Finally, the highest polymorphic amplicon was observed on primer locus "OPB-12" with an amplified value of 78.2%, while the least DNA polymorphic amplicon was observed on primer "OPH-15" with a value of 55.9% (Table 16).

Table 11. Qualitative description of nucleic acid extracted from *Auricularia* species in Lagos State.

Station	Code	Town	Latitude	Longitude	Nucleic acid conc. (ng/μL)	$A_{260/280}$
09	LA1	Ikeja	6.6180°N	3.3209°E	79.00	2.08
10	LA2	Ojo	6.4579°N	3.1580°E	282.3	2.14
11	LA3	Ikeja	6.4553°N	3.3641°E	309.1	2.12
12	LA4	Badagry	6.4316°N	2.8876°E	137.8	2.13
13	LA5	Epe	6.6055°N	3.9470°E	890.0	1.83
14	LA6	Shomolu	6.5392°N	3.3842°E	96.90	2.11
15	LA7	Ikorodu	6.6194°N	3.5105°E	94.00	2.11
16	LA8	Ikeja	6.5273°N	3.3414°E	187.7	2.03

LA: Lagos.

Table 12. Qualitative description of nucleic acid extracted from *Auricularia* species in Oyo State.

Station	Code	Town	Latitude	Longitude	Nucleic acid conc. (ng/μL)	$A_{260/280}$
17	OY1	Moniya	7.5249°N	3.9152°E	110.6	2.06
18	OY2	Egbeda	7.3796°N	3.9675°E	1507.5	1.92
19	OY3	Ido	7.5077°N	3.7194°E	96.70	2.07
20	OY4	Iseyin	7.9765°N	3.5914°E	1118	1.96
21	OY5	Ogbomosho	8.1227°N	4.2436°E	543.5	1.99
22	OY6	Idi Ayunre	7.2247°N	3.8732°E	193.8	2.10
23	OY7	Oyo	7.8430°N	3.9368°E	490.7	2.01
24	OY8	Igbeti	8.7699°N	4.1104°E	239.3	2.10

OY: Oyo.

Table 13. Qualitative description of nucleic acid extracted from *Auricularia* species in Ekiti State.

Station	Code	Town	Latitude	Longitude	Nucleic Acid Conc. (ng/μL)	$A_{260/280}$
25	EK1	Ado Ekiti	7.6124°N	5.2371°E	867.5	2.04
26	EK2	Iye	7.9591°N	5.2371°E	87.30	1.74
27	EK3	Ikole	7.7983°N	5.5145°E	80.80	1.75
28	EK4	Oye	7.7979°N	5.3286°E	120.3	1.95
29	EK5	Igede	7.7313°N	5.2476°E	100.6	2.11
30	EK6	Ikere	7.4991°N	5.2319°E	450.2	1.99
31	EK7	Ijero Ekiti	7.8120°N	5.0677°E	125.0	2.09
32	EK8	Emure Ekiti	7.4317°N	5.4621°E	138.0	2.39

EK: Ekiti.

Assessment of polymorphic nucleotide band formation

The profiled DNA amplicons showed little or no polymorphism at higher base pair units (Table 17). It was observed that none of the primers used in this research (except OPB-21 with 45/48 polymorphic DNA bands) had polymorphic bands at 900 bp. Subsequently, primers OPB-20 and OPB-21 were the only primers that registered polymorphism at 800 bp, that is, 46/48 and 9/48 polymorphic DNA bands, respectively. At 700 base pair units, five (5) out of the fourteen (14) primers investigated, were associated with polymorphic DNA activities, that is, OPB-11 (6/48 bands, as shown in Figure 4), OPB-12 (22/48 bands, as shown in Figure 5), OPB-20 (43/48 bands, as shown in Figure 7), OPB-21 (12/48 bands, as shown in Figure 8) and OPT-10 with 38/48 polymorphic DNA bands (Table 17). At lower base pair units, majority of the DNA primers were able to record DNA band polymorphism on electrophoresis gel.

Table 14. Qualitative description of nucleic acid extracted from *Auricularia* species in Ondo State.

Station	Code	Town	Latitude	Longitude	Nucleic acid conc. (ng/μL)	$A_{260/280}$
33	OD1	Idanre	7.0914°N	5.1484°E	190.6	2.03
34	OD2	Igbokoda	6.2585°N	4.7692°E	107.9	1.67
35	OD3	Ile Oluji	7.2825°N	4.8521°E	92.10	2.02
36	OD4	Ore	6.7519°N	4.8780°E	105.0	2.08
37	OD5	Okitipupa	6.5025°N	4.7795°E	309.1	2.12
38	OD6	Ifon	6.9235°N	5.7774°E	56.50	1.83
39	OD7	Owo	7.1989°N	5.5932°E	94.00	2.11
40	OD8	Igbara Oke	7.3877°N	5.0807°E	1507.5	1.62

OD: Ondo.

Table 15. Qualitative description of nucleic acid extracted from *Auricularia* species in Osun State.

Station	Code	Town	Latitude	Longitude	Nucleic acid conc. (ng/μL)	$A_{260/280}$
41	OS1	Ota Aiyebaju	7.5912°N	4.7329°E	543.5	1.99
42	OS2	Ejigbo	7.9045°N	4.3052°E	490.7	2.01
43	OS3	Oke Ila Orangun	7.9946°N	4.9974°E	867.5	2.04
44	OS4	Ikirun	7.9227°N	4.6347°E	87.30	1.75
45	OS5	Ila Orangun	8.0121°N	4.8988°E	80.80	1.85
46	OS6	Ilobu	7.9021°N	4.5315°E	120.3	1.95
47	OS7	Iwo	7.6292°N	4.1872°E	100.6	2.11
48	OS8	Ibokun	7.8019°N	4.7692°E	193.8	2.10

OS: Osun.

At 600 bp, eight (8) primers recorded polymorphic activities on electrophoresis gel, that is, OPB-11 (13/48 bands), OPB-12 (2/48 bands), OPB-15 (2/48 bands), OPB-21 (37/48 bands), OPT-1 (9/48 bands), OPT-10 (41/48 bands) and OPT-19 (30/48 bands, as shown in Figure 10). At 500 bp, ten (10) DNA primers, including OPB-15 with 26/48 DNA bands (Figure 6), recorded polymorphic band formation, at 400 bp, thirteen (13) out of the fourteen (14) DNA primers recorded polymorphic activities, OPH-10 DNA primer showed the first sign of polymorphism at 400 bp with 3/48 DNA bands (Figure 9). Finally, at 300, 200, and 100 bp, all the primers used for this experiment showed significant levels of band formation when compared with the amplified DNA fragments from the wild *Auricularia* specimens (Table 17). The DNA primers OPB-20 and OPB-21 best described the nucleotide polymorphism with the investigated genome of the wild *Auricularia* specimens collected from the wild forests of Southwest, Nigeria.

The efficiency of the DNA primers used for this research

The DNA primers OPB-11, OPB-15, OPB-20, OPB-21, OPT-1 and OPT-5 were able to effectively detect 90 to 99% polymorphism in the DNA strands of the *Auricularia* specimen profiled on electrophoresis gel (Table 18), as such they were listed as the best markers for this research. Other DNA markers such as OPH-3, OPT-10 and OPT-19 showed an impressive 80 to 89% variability within the *Auricularia* population in Southwest Nigeria, while OPB-12 and OPH-5 were able to give between 70 and 79% variation in the examined *Auricularia* mushroom population. DNA markers OPH-10 and OPT-7 each gave between 60 and 69% variation, while OPH-15 was only able to detect between 10 and 19% variation at maximum in the *Auricularia* mushroom population of Southwest Nigeria (Table 18).

Grouping of Auricularia spp. in Nigeria based on genotype

The population of all the *Auricularia* mushrooms currently present in the six (6) states of Southwest Nigeria were effectively classified into six (6) clusters on the genetic dissimilarity chart (Figure 11) using PCR and RAPD markers on representative samples collected during field survey. The six (6) clusters of mushroom categories were effectively characterized into three (3) distinct species and further sub-classified into five (5) cultivars (sub-

Table 16. Genetic information for the amplified Auricularia DNA samples.

DNA primers	Major allele frequency	No. of allele	Genetic diversity	PIC	Polymorphic Amplicons (%)
OPB-11	0.44	14.0	0.78	0.76	76.2
OPB-12	0.40	13.0	0.80	0.78	78.2
OPB-15	0.35	11.0	0.79	0.76	76.4
OPB-20	0.44	14.0	0.78	0.76	76.3
OPB-21	0.54	16.0	0.69	0.68	67.9
OPH-03	0.46	12.0	0.75	0.74	73.6
OPH-05	0.56	6.0	0.63	0.60	60.1
OPH-10	0.44	5.0	0.72	0.68	67.9
OPH-15	0.60	5.0	0.59	0.56	55.9
OPT-01	0.46	11.0	0.74	0.71	71.3
OPT-05	0.54	8.0	0.65	0.62	62.0
OPT-07	0.46	7.0	0.72	0.69	68.7
OPT-10	0.46	16.0	0.76	0.75	75.4
OPT-19	0.52	14.0	0.70	0.69	68.7
Average	0.48	10.9	0.72	0.70	70.0

Sample size (n = 48).

Table 17. DNA polymorphism on electrophoresis gel (RAPD).

Marker	Polymorphic Nucleotide Count/bp Units								
	900	800	700	600	500	400	300	200	100
OPB-11	-	-	6	13	13	6	33	38	46
OPB-12	-	-	22	2	31	37	24	32	32
OPB-15	-	-	-	32	26	21	10	42	46
OPB-20	-	46	43	2	35	16	2	32	44
OPB-21	45	9	12	37	17	9	1	9	39
OPH-3	-	-	-	-	36	27	14	27	40
OPH-5	-	-	-	-	-	-	36	35	4
OPH-10	-	-	-	-	-	3	21	31	7
OPH-15	-	-	-	-	-	3	4	7	5
OPT-1	-	-	-	9	44	28	9	38	33
OPT-5	-	-	-	-	-	2	10	44	17
OPT-7	-	-	-	-	2	2	4	26	31
OPT-10	-	-	38	41	15	12	5	4	26
OPT-19	-	-	-	30	38	41	16	12	5

OS1, OS2, OS3, OS4, OS5, OS6, OS7, OS8, EK1, EK2, EK3, EK4, EK5, EK6, EK7, EK8
Cultivar II (Group V): OY2, LA1, LA2, LA3, LA4, OY5, OY6, OY7, OY8

Species 3: Unknown (Outliers)

OY3, OY4

The farther apart the *Auricularia* mushrooms on the genetic dissimilarity tree, the more related the species.

DISCUSSION

Thirty-one (31) locations were mapped in the wild where *A. auricula* can be found in the forest of Southwestern Nigeria. The geospatial analysis conducted showed that *A. auricula* was dominant in the forest of Ekiti state within the fringes of Ado Ekiti, Iye, Ikole, Oye, Igede, Ikere, Ijero Ekiti, and Emure Ekiti. Also, the same species of *Auricularia* was evenly distributed around the forest vegetation of Ota Aiyebaju, Ejigbo, Oke Ila Orangun, Ikirun, Ila Orangun, Ilobu, Iwo, and Ibokun (in Osun State), Ifo, Ijebu Ode, Ikenne, Shagamu, Odeda, and

Table 18. The efficiency of the DNA primers in electrophoresis gel analysis at varying "bp-units".

Marker	DNA polymorphism (%), bp								
	900	800	700	600	500	400	300	200	100
OPB-11	0.0	0.0	12.5	27.1	27.1	12.5	68.8	79.2	95.8
OPB-12	0.0	0.0	45.8	4.2	64.6	77.1	50.0	66.7	66.7
OPB-15	0.0	0.0	0.0	66.7	54.2	43.8	20.8	87.5	95.8
OPB-20	0.0	95.8	89.6	4.2	72.9	33.3	4.2	66.7	91.7
OPB-21	93.8	18.8	25.0	77.1	35.4	18.8	2.1	18.8	81.3
OPH-3	0.0	0.0	0.0	0.0	75.0	56.3	29.2	56.3	83.3
OPH-5	0.0	0.0	0.0	0.0	0.0	0.0	75.0	72.9	8.3
OPH-10	0.0	0.0	0.0	0.0	0.0	6.3	43.8	64.6	14.6
OPH-15	0.0	0.0	0.0	0.0	0.0	6.3	8.3	14.6	10.4
OPT-1	0.0	0.0	0.0	18.8	91.7	58.3	18.8	79.2	68.8
OPT-5	0.0	0.0	0.0	0.0	0.0	4.2	20.8	91.7	35.4
OPT-7	0.0	0.0	0.0	0.0	4.2	4.2	8.3	54.2	64.6
OPT-10	0.0	0.0	79.2	85.4	31.3	25.0	10.4	8.3	54.2
OPT-19	0.0	0.0	0.0	62.5	79.2	85.4	33.3	25.0	10.4

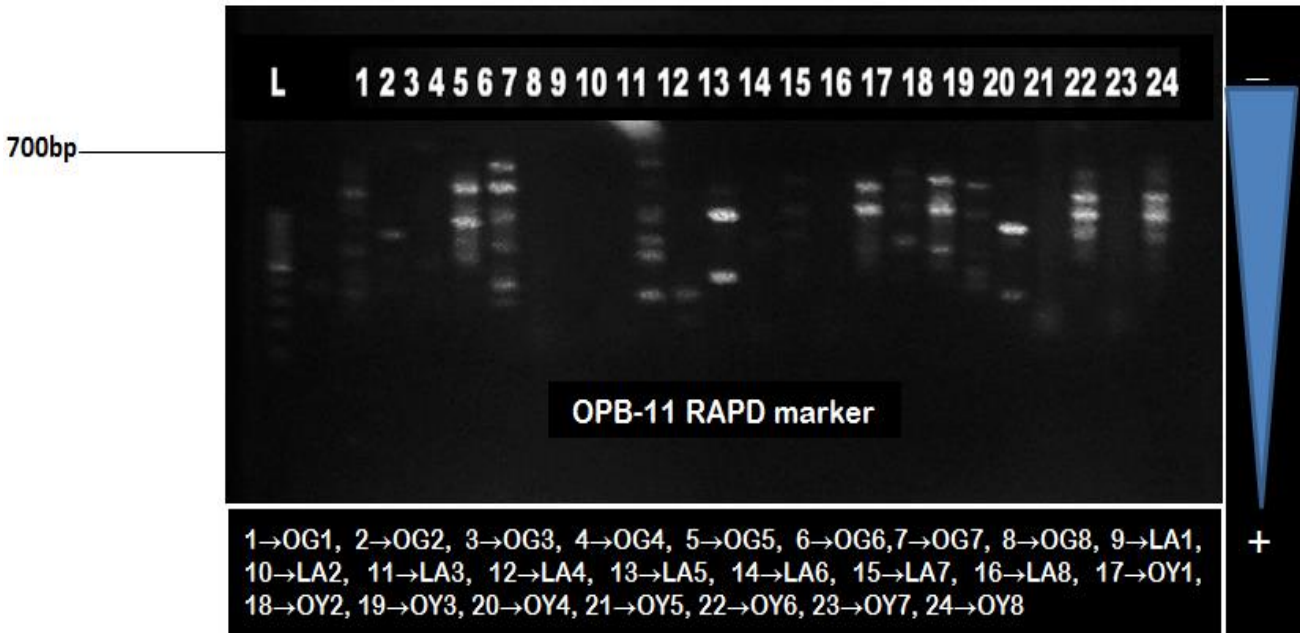

Figure 4. Electrophoresis gel analysis of wild *Auricularia* genome against OPB-11 DNA primer.

Odogbolu (in Ogun State), Egbeda, Ogbomosho, Idi Ayunre, Oyo, and Igbeti (in Oyo State) and Agege, Ojo, Apapa, Badagry, Epe, Shomolu, Ikorodu and Mushin (in Lagos State). There was no trace of *A. auricula* in the forest vicinity of Ondo State as at the time of filing this report. The unusual absence of this species of *Auricularia* in the rainforest and Savannah vegetations of Ondo State might be due to over exploration of this particular species for food and folklore medicine by the indigenous people of Ondo State, or maybe due to unfavourable weather conditions. In any case, the rationale behind the total absence of this species in Ondo State is still unclear and more scientific probing should be setup to explain this observation. The findings of this study were in concordance with the report of Adeniyi et al. (2018), who identified the carpophores of *A. auricula-judae* in ligneous habitat in Southwest Nigeria.

In contrast to the observation noted for *A. auricula* in Ondo State, *A. polytricha* was found in abundance in the forest terrain and savannah region of Ondo State, within

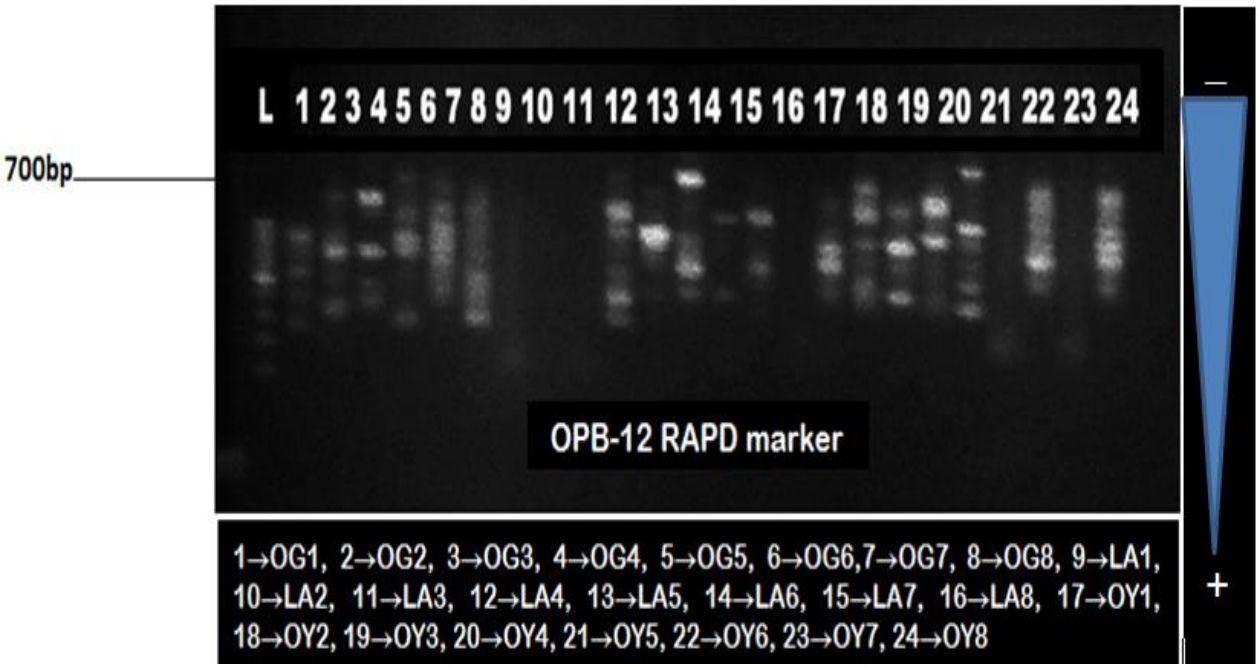

Figure 5. Electrophoresis gel analysis of wild *Auricularia* genome against OPB-12 DNA primer.

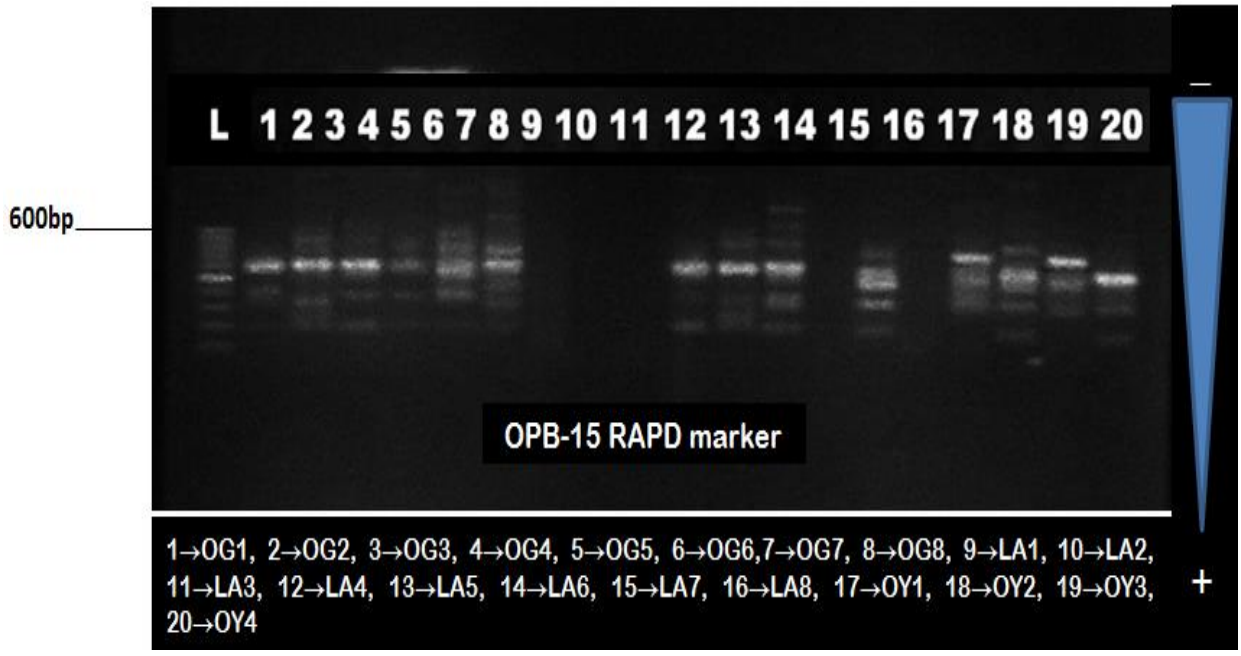

Figure 6. Electrophoresis gel analysis of wild *Auricularia* genome against OPB-15 DNA primer.

the environs of Idanre, Igbokoda, Ile Iluji, Ore, Okitipupa, Ose, Owo, and Ifedore, respectively. It was sparsely distributed in the fringes of Lagos State, in places like Shomolu, Ikorodu and Ikeja only. Unfortunately, this species of *Auricularia* was neither found in the forest of Ogun, Ekiti, Osun, nor Oyo states. This strange observation is a puzzle that requires answer. The observation noted in the current study was in line with the research by Titilawo et al. (2019) who collected samples of *A. polytrichia* from the ligneous (woody) substrates within the forest of Southwestern Nigeria, in order to determine its nutritional (proximate) and phytochemical

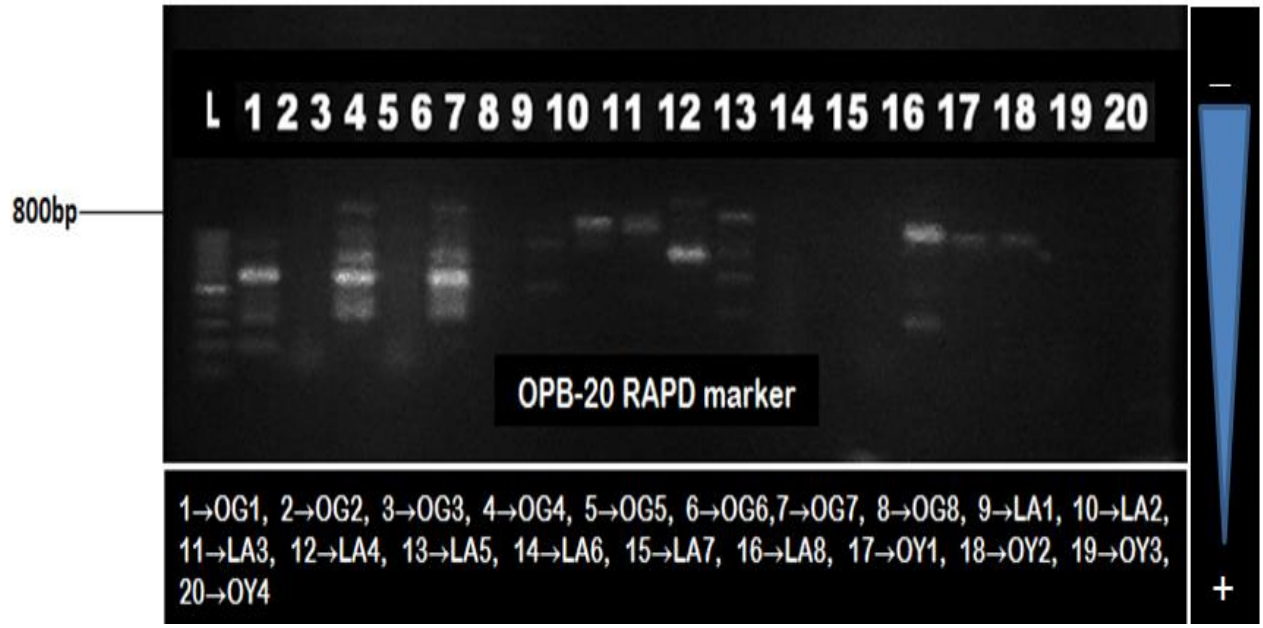

Figure 7. Electrophoresis gel analysis of wild *Auricularia* genome against OPB-20 DNA primer.

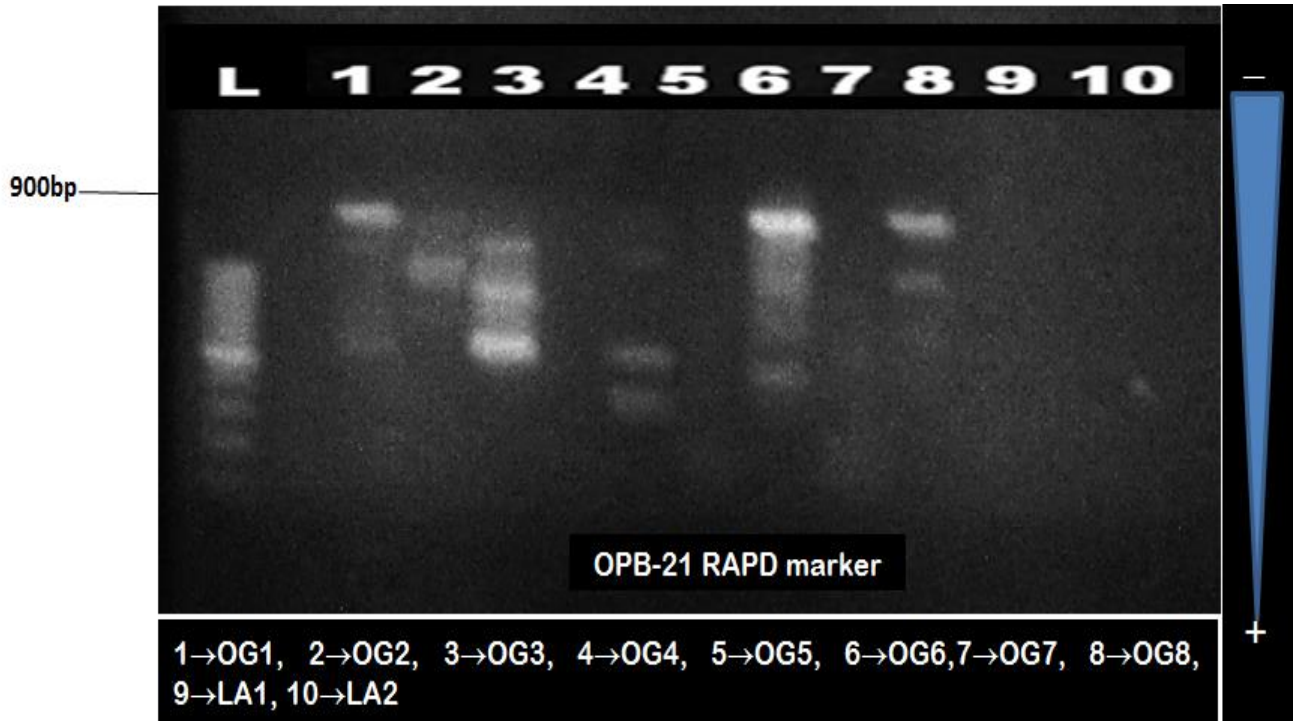

Figure 8. Electrophoresis gel analysis of wild *Auricularia* genome against OPB-21 DNA primer.

properties. Further probing of the already identified *Auricularia* spp. was carried out using genome similarity aided by PCR and electrophoresis gel techniques (RAPD). The analysis showed that there was slight variation in the genetic makeup of the *Auricularia* (mushrooms) found in Southwestern Nigeria. The group was further subdivided into five (5) cultivars (sub-species), based on the genome similarity index. The

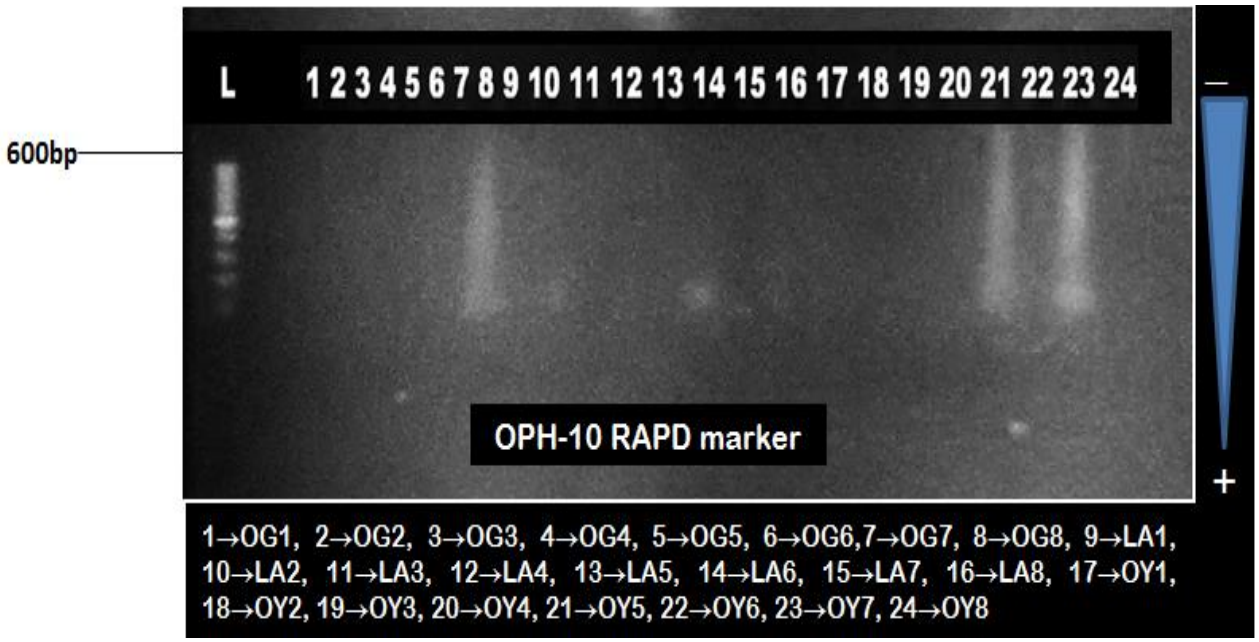

Figure 9. Electrophoresis gel analysis of wild *Auricularia* genome against OPH-10 DNA primer.

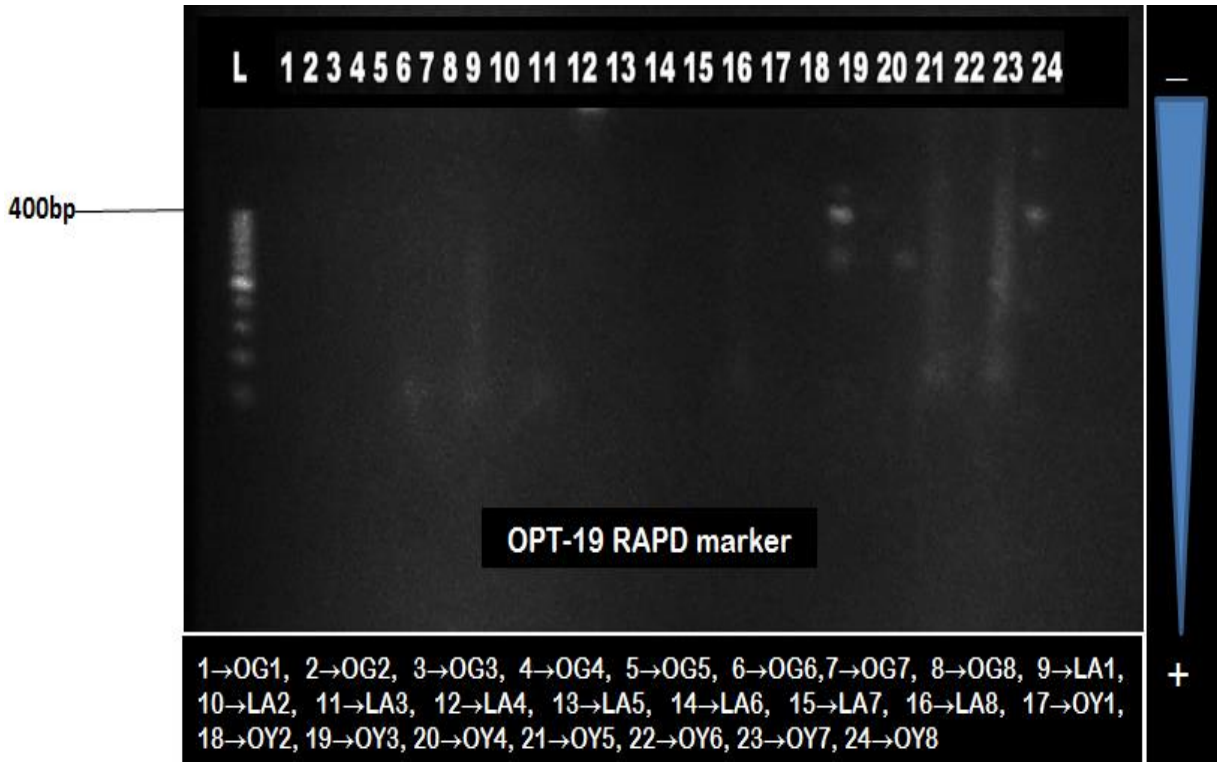

Figure 10. Electrophoresis gel analysis of wild *Auricularia* genome against OPT-19 DNA primer.

taxonomic groups unearthed by this research are still under review. The findings from this research were supported by the research work of Ekun et al. (2018), who earlier reported a similar observation of an unidentified Auricularia (mushroom) found deep in the tropical rainforest of Southwest Nigeria.

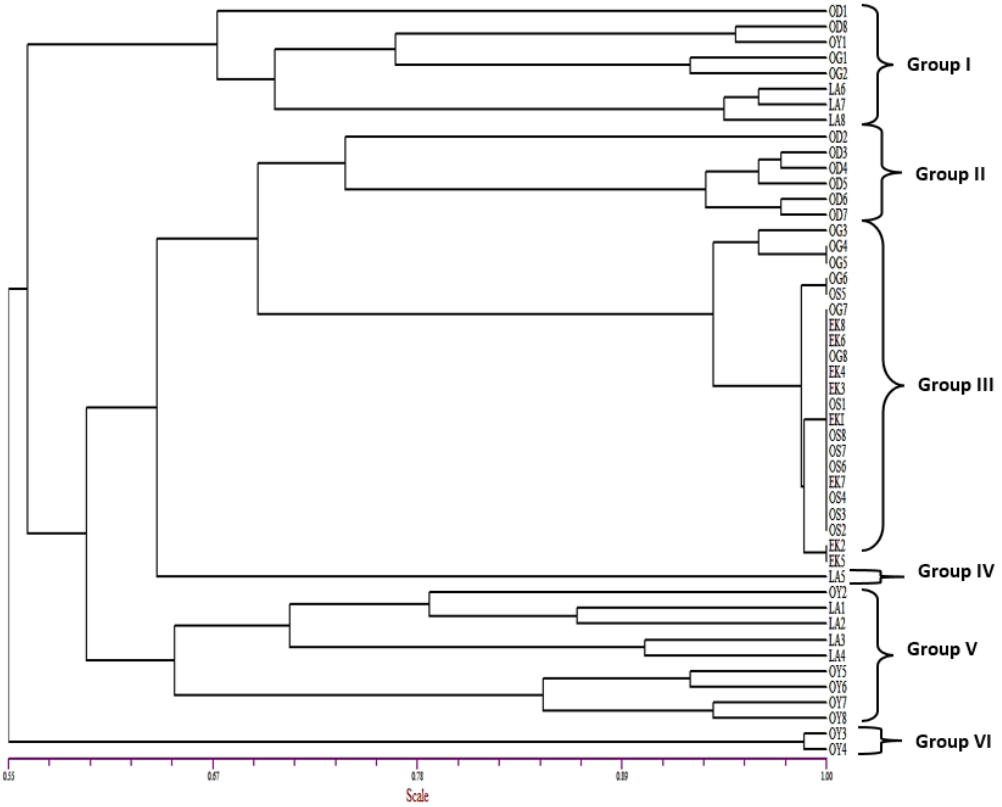

Figure 11. Genetic dissimilarity among the population of *Auricularia* spp. in Southwestern Nigeria.

Conclusion

The study was able to establish the fact that the population of *A. polytricha* in the wild forests and grassland vegetations of Southwest Nigeria are on the decline and that efforts geared towards rescuing and conservation of the germplasm of that particular *Auricularia* spp. should be put in place soonest. Also, an interesting development unravelled by this research was the collection of rare species of *Auricularia* mushrooms; although, these rare species are few and sparsely distributed around Southwest Nigeria, it is also pertinent that they are protected and conserved too. Laws involving selective or restricted mushroom poaching or foraging in the rural areas of Nigeria should be promulgated so that some of these endangered mushroom species can be protected in their natural habitat.

CONFLICT OF INTERESTS

The authors have not declared conflict of interests.

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Full Length Research Paper

Antioxidative and anti-aging activities of *Abeliophyllum distichum* Nakai extracts fermented with *lactobacillus plantarum* and *lactobacillus brevis*

KiBeom Lee[1*], Ju Hyun Park[2] and Yun Sung Kim[2]

[1]Bioindustry Center, Incheon Technopark, 12 Gaetbeol-ro, Yeonsu-gu, Incheon, South Korea.
[2]Genetrone Biotech Co., Ltd, 2F, 4F, Sehyun Building, 15 Hongsanbuk-ro, Wansan-gu, Jeonju-si Jeollabuk-do, South Korea.

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***Abeliophyllum distichum* Nakai is a deciduous shrub that belongs to Oleaceae Abeliophyllum and grows only in Korea. In this study, the antioxidative activity, cytotoxicity, anti-aging activity, and levels of acteoside and isoacteoside were compared between non-fermented and fermented (using *Lactobacillus plantarum* and *Lactobacillus brevis*) extracts of *A. distichum* Nakai. The cytotoxicity of these extracts, along with their ability to inhibit elastase activity and Matrix metalloproteinase-1 (MMP-1) expression, and their ability to promote type I procollagen synthesis were investigated in human dermal fibroblasts cells. These tests revealed that the fermented extract possessed higher antioxidant and anti-aging activities compared with the non-fermented extract. The levels of acteoside and isoacteoside were about 1.25 and 1.05 times higher in the fermented extract than in the non-fermented extract. It was speculated that they were converted from acteoside glucosides and isoacteoside glucosides via bioconversion by the fermentation strains. Together, these findings indicate that extracts of *A. distichum* Nakai show good potential as antioxidative and anti-aging cosmetic materials.**

Key words: *Abeliophyllum distichum* Nakai, *Lactobacillus plantarum, Lactobacillus brevis,* fermentation, antioxidative activity, anti-aging activity.

INTRODUCTION

Wrinkles naturally occur as skin ages, but the photoaging caused by ultraviolet (UV) rays of sunlight further reduces skin elasticity and increases wrinkles. The active oxygen species generated by UV rays greatly influence this process. For example, active oxygen inhibits the production of collagen, which helps maintain skin elasticity (Kim et al., 2007). Oxidative stress induced by active oxygen increases the production of MMPs, which degrade collagen; leads to decreased skin elasticity and increases wrinkles (Pentland et al., 1995). Therefore, a substance that excels at antioxidation can be a good means for improving skin wrinkles. Various substances are currently being tested with this goal in mind. Many groups are focusing on natural materials, which are expected to be stimulating the skin compared to synthetic materials.

Most of the natural plant extracts used as cosmetic materials are extracted as glycosides. Recent studies

*Corresponding author. E-mail: klee02@empas.com.

have focused on converting the glycosides of natural extracts into active materials by using fermentation (Huynh et al., 2016; Wang et al., 2016; Lee and Paik, 2017), which can reduce the size and polarity of the molecules to improve their skin absorption and efficacy (Lee et al., 2014; Hong and Han, 2002; Hirbumi et al., 1998).

Abeliophyllum distichum Nakai is a rare species that is found only in Korea. This member of family Oleaceae is a deciduous broad-leaved shrub that is so rare; it has been designated as a natural monument (Lee et al., 2014; Hong and Han, 2002). Recently, however, it has been mass-produced and grown in the Goesan area of Chungbuk province. Studies on *A. distichum* Nakai extracts have examined their antioxidant and anti-aging efficacy (Hirbumi et al., 1998; Kim and Lee, 2015). The extracts have been found to contain acteoside and isoacteoside, which are phenylpropanoid glycosides that reportedly have antioxidant and anti-aging effects (Yoon et al., 2009).

Most of the previous studies have focused on non-fermented extracts of *A. distichum* Nakai. Here, the cytotoxicity, antioxidative and anti-aging activity, and levels of acteoside and isoacteoside in extracts of *A. distichum* Nakai were compared with and without fermentation using *Lactobacillus plantarum* and *Lactobacillus brevis*.

MATERIALS AND METHODS

Preparation of *A. distichum* Nakai fermented extracts

A. distichum Nakai leaves were collected from a natural habitat in South Korea, Goesan area of Chungbuk province in February 2019. *A. distichum* Nakai leaves were dried at room temperature and subjected to extraction. The distilled water extract of *A. distichum* Nakai (the non-fermented sample) was obtained using 20 volumes of water at 95°C for 18 h. *L. plantarum* and *L. brevis* strains were inoculated to De Man, Rogosa and Sharpe (MRS) broth and grown at 37°C for 24 h. For fermentation, the *A. distichum* Nakai solution (3%) was inoculated with fresh bacterial subculture (4% v/v), incubated at 37°C for 24 h, and subjected to sterilization and filtration. The filtered solution of fermented sample was concentrated and spray-dried.

Measurement of antioxidant activity of the extracts

The antioxidant capacity of extracts was evaluated by measuring free radical scavenging activity using the DPPH assay (Widowati et al., 2003). Samples were prepared at concentrations of 0.1, 0.25, 0.5, and 1.0 mg/ml, with the non-fermented and fermented extracts. After incubation at room temperature for over 30 min, free radical scavenging activity was determined by mixing with 500 μM DPPH solution (1:1) and incubating in the dark, followed by measurement of absorbance at 517 nm using a spectrophotometer.

Measurement of the elastase inhibitory activity

The elastase inhibitory assay was performed as previously described (Thring et al., 2009). Briefly, 20 μl volumes containing various concentrations of non-fermented or fermented extracts, 10 μl elastase from porcine pancreas (2.5 units), and 125 μl Tris buffer were preincubated for 15 min at 25°C, mixed with 0.1 mM N-succinyl-Ala-Ala-Ala-*p*-nitroanilide substrate (20 μl), and incubated for 10 min at 25°C. Absorbance was measured at 410 nm.

Analysis of cytotoxicity of the extracts

Human dermal fibroblasts (Hs68 cells, CRL 1635; American Type Culture Collection, Rockville, MD) were screened for their cytotoxicity following exposure to the various extracts. The MTT assay (Sigma-Aldrich) was used to determine cell viability. Briefly, Hs68 cells were seeded to 96-well plates, grown to 60~80% confluence, treated with 200 μl medium containing various concentrations of non-fermented and fermented extracts at a range of concentrations, and incubated at 37°C for 24 h. Each well was treated with MTT reagent (20 μl), the plate was incubated for an additional 1 h, and absorbance was read at 570 nm using a microplate reader.

Enzyme-linked immunosorbent assay (ELISA)

For analysis of MMP-1 and type I procollagen, Hs68 cells were seeded to a 24-well plate (5×10^4 cells/well) in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were grown for 24 h treated with various concentrations of the non-fermented and fermented extracts in FBS-free medium for 24 h, cultured under 37°C in 5% CO_2 for an additional 24 h, and treated with 10 ng/ml tumor necrosis factor α (TNF-α). The culture media were collected and the immunoreactivities of MMP-1 and type I procollagen were measured by ELISA using commercially available kits, followed by measurement of absorbance at 450 nm.

Component analysis

HPLC was performed using an Agilent 1200 series gradient HPLC system. Briefly, fermented or non-fermented extracts dissolved in methanol (1 mg/ml) were injected (20 μl) onto a reverse-phase column (Agilent-Eclipse Plus C18, 5 μm, 4.9 × 150 mm). The mobile phase was a mixture of water (A) and methanol (B) and progressed from 10 to 90% B over a period of 50 min at a flow rate of 1 ml/min.

Statistical analysis

All data are presented as mean ± standard deviation of three replicates. Differences among treatments were assessed by analysis of variance (ANOVA), followed by Dunnett's test. *p*-value of <0.05 was regarded as significant.

RESULTS AND DISCUSSION

DPPH radical scavenging activity of non-fermented and fermented *A. distichum* Nakai extracts

The scavenging activities of non-fermented and fermented *A. distichum* Nakai extracts increased dose-dependently (Figure 1). The non-fermented extract

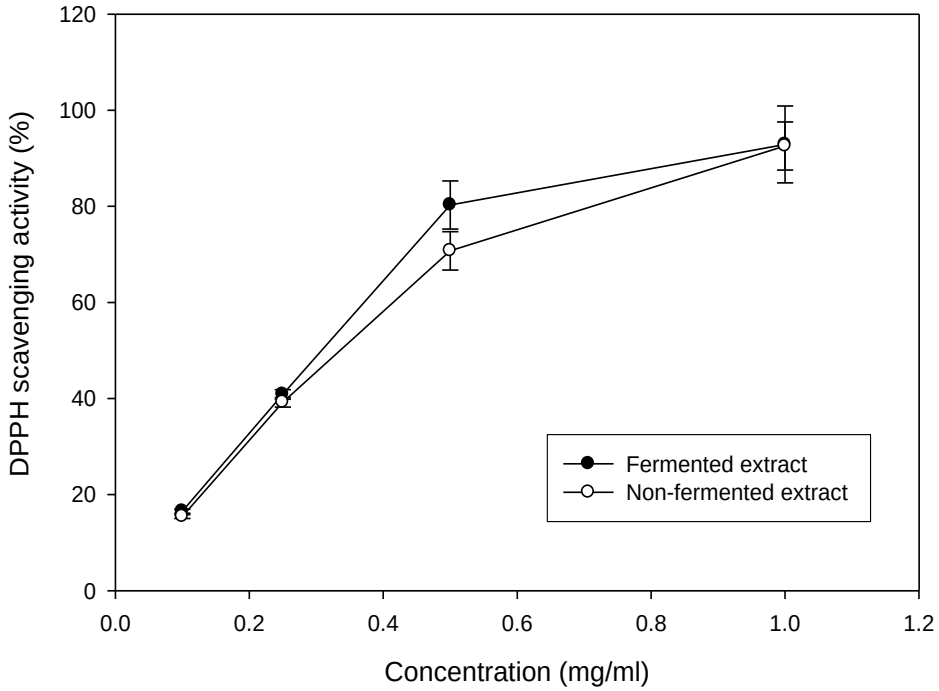

Figure 1. Free radical DPPH scavenging activity of the non-fermented and fermented extracts. Data are presented as the mean ± SD of three independent experiments. Means not sharing a common letter were significantly different at $p<0.05$.

exerted inhibitory effects of 15.43, 39.20, 70.74 and 92.55% at concentrations of 0.1, 0.25, 0.5, and 1.0 mg/ml, respectively, while the same doses of fermented extract exerted inhibitory effects of 16.54, 40.85, 80.27 and 92.87%, respectively. This indicates that fermented *A. distichum* Nakai extract showed higher scavenging activity for DPPH radicals at the concentrations tested. The IC_{50} values were 0.40 and 0.37 mg/ml for non-fermented and fermented *A. distichum* Nakai extracts, respectively.

Cytotoxicity effects

Many lactic acid bacteria-fermented products are known to have various regulatory functions, including anti-diarrheal, antiviral, anti-allergy, and immune effects (Ayivi et al., 2020). Since these fermented products tend to be non-cytotoxic, they have long been ingested as functional foods (Sanders and Huis, 1999). To assess the effect of the non-fermented and fermented extracts on cell viability, fibroblasts cells were herein treated with 0~200 μg/ml of the non-fermented and fermented extracts, and the MTT assay was performed. As shown in Figure 2, significant toxicity was observed within the tested concentration range. This indicates that, consistent with the literature, the lactic acid bacteria-fermented extract did not appear to exert cytotoxicity.

Elastase inhibitory activity

Reduced skin elasticity and decreased elastase activity are important components of wrinkle formation. The elastase inhibitory activity (IC_{50}) of the non-fermented extract was 147.30 μg/ml and that of the fermented extract was 134.57 μg/ml, as shown in Figure 3. Both extracts had lower IC_{50} values than Epigallocatechin gallate (EGCG) (IC_{50} 25.3 μg/ml), which was used as a reference compound. The IC_{50} of the fermented extract was approximately 8.64% higher than that of the non-fermented extract, indicating that the elastase inhibitory activity was increased by fermentation. Studies on the elastase inhibitory activity of many plants have been reported (Thring et al., 2009; Moon et al., 2010). However, this is the first examination of the elastase inhibitory activity of the fermented extract of *A. distichum* Nakai. Additional research will be needed to identify the compound(s) that confer(s) this activity in the fermented product of *A. distichum* Nakai.

MMP-1 inhibitory activity

MMP-1 is a protease that specifically acts on collagen; inhibitors of MMP-1 inhibit collagen degradation and thus help maintain skin elasticity and prevent wrinkle formation (Park et al., 2008). Here, we measured the secretion of

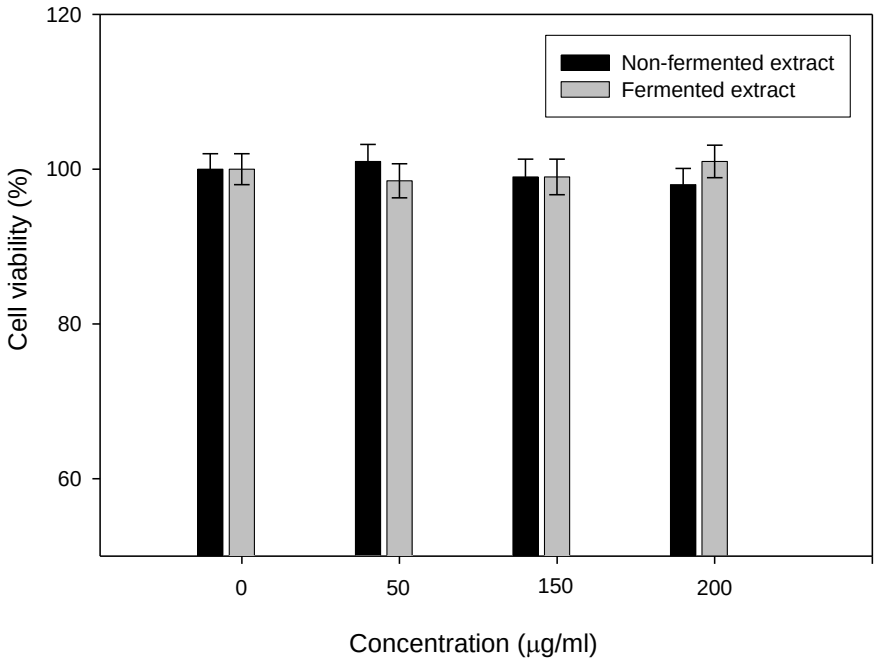

Figure 2. Viability of cells treated with non-fermented and fermented extracts, as assessed by MTT assay. Data are presented as the mean ± SD of three independent experiments. Means not sharing a common letter were significantly different at $p<0.05$.

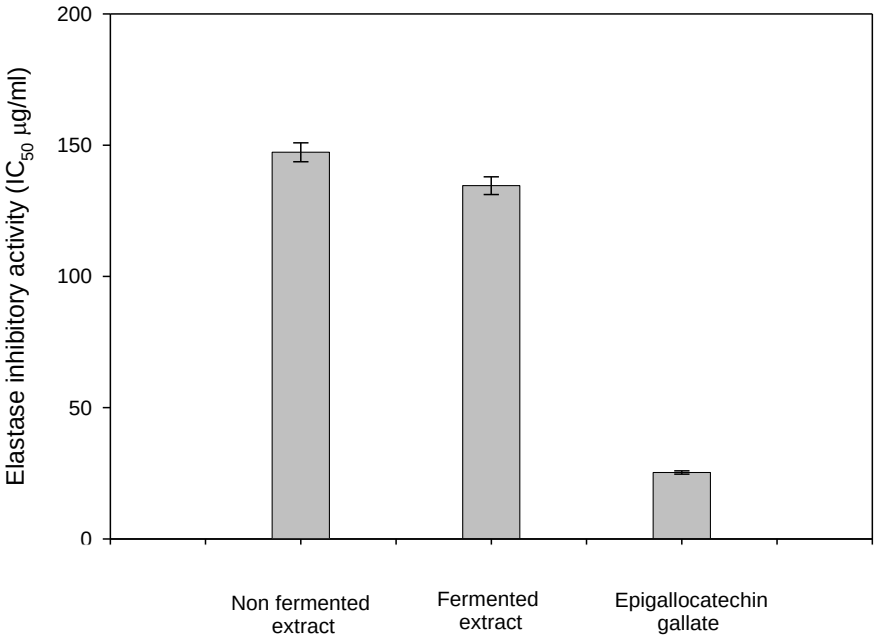

Figure 3. Elastase inhibition by the non-fermented and fermented extracts. Data are presented as the mean ± SD of three independent experiments. Means not sharing a common letter were significantly different at $p<0.05$.

MMP-1 from cells treated with the non-fermented or fermented *A. distichum* Nakai extracts. As shown in Figure 4, the results confirmed the potential wrinkle-improving activity of these extracts, as MMP-1 production

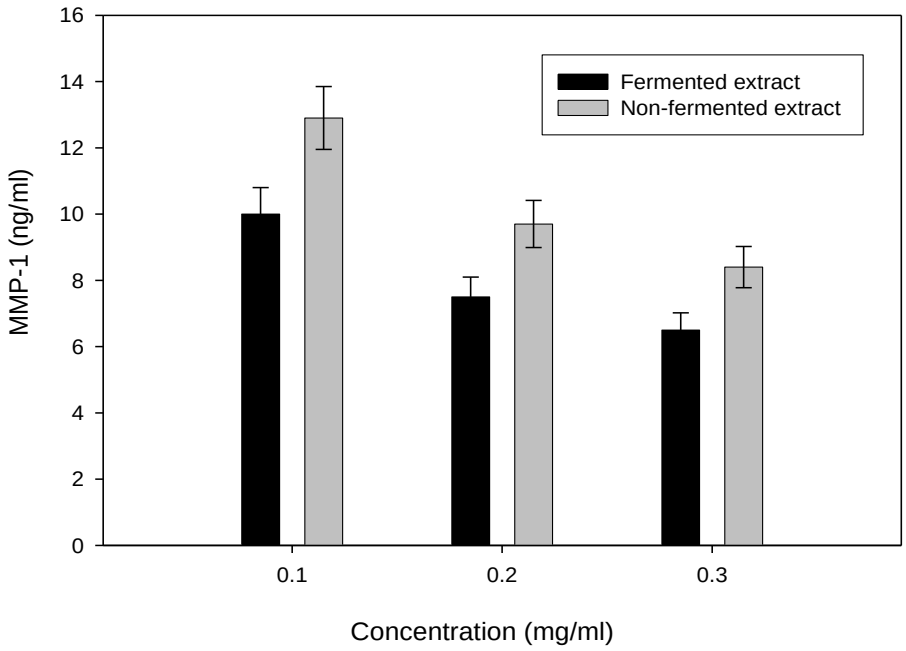

Figure 4. The inhibitory effect of the fermented and non-fermented extracts on MMP-1 production. Data are presented as the mean ± SD of three independent experiments. Means not sharing a common letter were significantly different at $p<0.05$.

was decreased by increasing concentrations of both fermented and non-fermented extracts. Treating cells with 0.1, 0.2, and 0.3 mg/ml of the non-fermented extract inhibited the MMP-1 level by 12.9, 9.7, and 8.4 ng/ml, respectively, whereas treatment of cells with the same concentrations of the fermented extract inhibited the MMP-1 level by 10, 7.5, and 6.5 ng/ml, respectively. These results suggest that the fermented extract has a stronger ability to inhibit MMP-1 production, compared to the non-fermented extract. The mechanism by which the fermented extract additionally suppresses MMP-1 production warrants future study.

Effects on type I procollagen production

It was also evaluated whether non-fermented and fermented *A. distichum* Nakai extracts influenced the production of type I procollagen. Procollagen contains a peptide base sequence called a propeptide at the amino terminus and carboxy terminus. The propeptide is known to facilitate the folding of the procollagen molecule in the endoplasmic reticulum; it is cleaved and separated from the collagen molecule when the collagen-polymerization reaction occurs (Park et al., 2019). Accordingly, the degree of collagen biosynthesis in a cell can be determined by measuring the amount of the separated propeptide (Parfitt et al., 1987). As shown in Figure 5, it was found that treating cells with 0.1, 0.2, and 0.3 mg/ml of the fermented extract increased the type I procollagen production by 22.4, 28.1, and 33.7 ng/ml, respectively, whereas the same doses of non-fermented extract increased this production by 20, 25, and 30 ng/ml, respectively. These results suggest that type I procollagen production is induced more effectively by the fermented extract compared to the non-fermented extract.

Component analysis

HPLC was used to examine the contents of acteoside and isoacteoside in the fermented and non-fermented extracts. Two prominent peaks were observed within the 20-min analysis time (Figure 6). The retention times of peaks 1 and 2 in the HPLC chromatogram were identical to those of the standards for acteoside and isoacteoside, respectively, and thus the peaks were identified as corresponding to these proteins. The contents of acteoside and isoacteoside were 1.25 and 1.05 times higher, respectively, in the fermented extract compared to the non-fermented extract. The amounts of acteoside and isoacteoside were determined to be 372 and 429 mg/100 g for the fermented extract and 298 and 450 mg/100 g for the non-fermented extract. Thus, these components, which were presumably bioconverted via the β-glucosidase activity of *Lactobacillus* strains undergoing fermentation, were increased in the fermented extract. This may explain the apparent improvements in the antioxidative and anti-aging potential of the fermented extract.

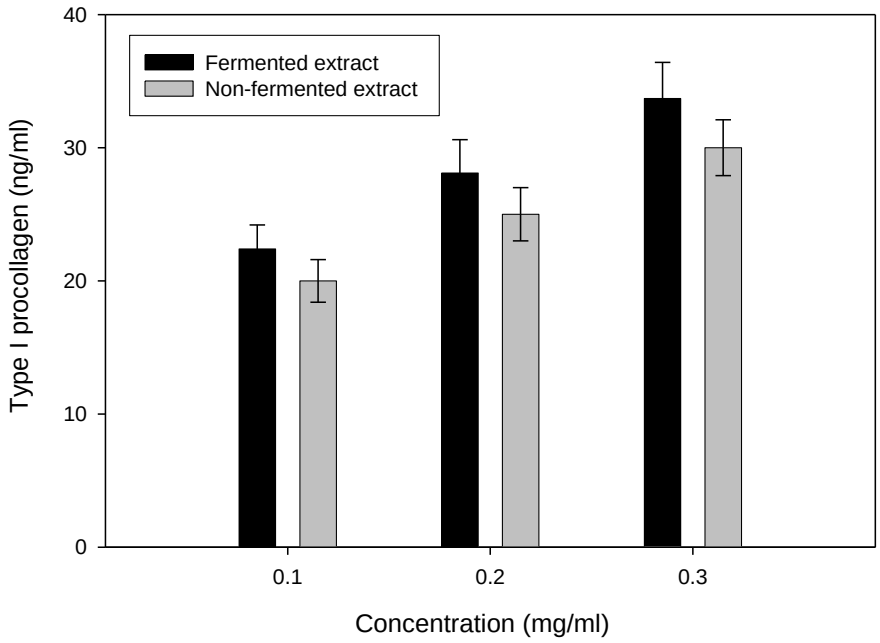

Figure 5. Type 1 procollagen synthesis induced by fermented and non-fermented extracts of *Abeliophyllum distichum* Nakai. Data are presented as the mean ± SD of three independent experiments. Means not sharing a common letter were significantly different at $p<0.05$.

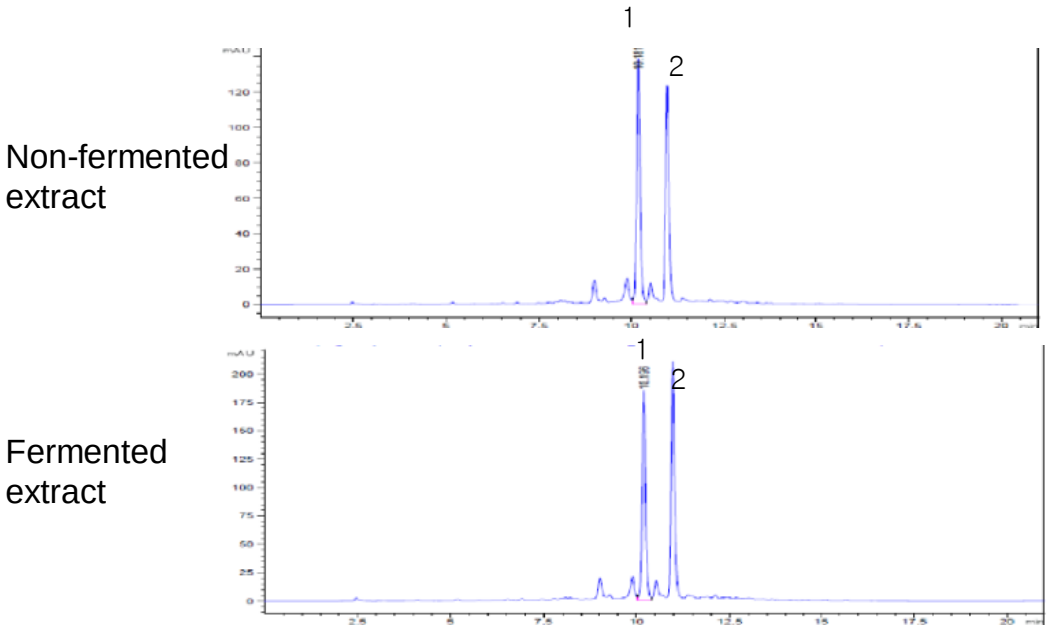

Figure 6. HPLC profiles of non-fermented and fermented extracts of *Abeliopyllum distichum* Nakai (1: acteoside; 2: isoacteoside).

In conclusion, it was observed that the fermented extract from *A. distichum* Nakai more effectively inhibits MMP-1 expression compared to the non-fermented extract. The results suggest that the fermented extract more effectively inhibits intracellular reactive oxygen species (ROS) production and may be useful as an anti- aging

substance for cosmetic applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antioxidant effects of ginger, garlic and onion aqueous extracts on 2-thiobarbituric acid reactive substances (2-TBARS) and total volatile basic nitrogen (TVB-N) content in chevon and pork during frozen storage

Lesten Eliez Chisomo Chatepa[1*], Kingsley George Masamba[2] and Jonathan Tanganyika[3]

[1]Basic Science Department, Faculty of Agriculture, Lilongwe University of Agriculture and Natural Resources, Bunda Campus, P. O. Box 219, Lilongwe, Malawi.
[2]Food Science and Technology Department, Faculty of Food and Human Sciences, Lilongwe University of Agriculture and Natural Resources, Bunda Campus, P. O. Box 219, Lilongwe, Malawi.
[3]Animal Science Department, Faculty of Agriculture, Lilongwe University of Agriculture and Natural Resources, Bunda Campus, P. O. Box 219, Lilongwe, Malawi.

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The study investigated the effect of 10% aqueous extracts of ginger, garlic and onion on the quality and shelf life of frozen chevon and pork as reflected by changes in values of 2-thiobartituric acid reactive substances (2-TBARS) and total volatile basic nitrogen (TVB-N) over a 14 day storage under frozen conditions. The pH, 2-TBARS and TVB-N of chevon and pork were measured from frozen storage at -20°C after 14 days. The 10% aqueous extracts resulted in low pH values of 5.63, 5.79 and 5.67 at 14 day for chevon mixed with ginger, garlic and onion, respectively. After 14 days of frozen storage, results on 2-TBARS expressed as mg MDA/kg meat, for chevon mixed with aqueous extracts of ginger, garlic and onion were 2.62±0.01, 1.45±0.01 and 4.71±0.03 which were lower compared to 5.93±0.01 for raw chevon. On the other hand, on day 14 of frozen storage, pork mixed with garlic aqueous extracts had lower TBARS value of 2.13±0.01 compared to the values obtained in pork mixed with onion, ginger and control pork sample, respectively. TVB-N, in mg/100 g, was highest in control raw chevon and pork registering values of 95.70±0.32 and 84.00±0.40, respectively after 14 days. Low values of TVB-N of 7.24±0.23, 12.37±0.23 and 16.61±0.50 were observed in chevon mixed with ginger, garlic and onion compared to the values for pork mixed with ginger, garlic and onion. Therefore, findings from this study have revealed that natural aqueous antioxidant extracts of ginger, garlic and onion has antioxidative effect on lipid peroxidation in frozen stored fresh chevon and pork.

Key words: 2-Thiobarbituric acid reactive substances (2-TBARS), total volatile basic nitrogen (TVB-N), antioxidants, pork, goat meat (chevon), ginger, garlic, onion.

INTRODUCTION

Global financial value of the meat sector is projected to be at USD 1142.9 billion by 2023 from an estimated value of USD 945.7 billion in 2018 (Shahbandeh, 2019). In 2020, global meat consumption was estimated at 360

million tons yearly with an increase of 58% in over the last decades. It is reported that population growth has caused 54% and the other 4% fraction comes from consumption per capita and increased consumers' income (Whitnall and Pitts, 2019). The upsurge in the global meat production and consumption is defined by the high nutritive value which is significant for good human health (Ruban, 2009).

Meat and meat based foods have high nutrient contents like vitamins, proteins, fats and minerals. Meat has high amount of n-polyunsaturated fatty acids and linoleic acid which are significant to human health (Ruban, 2009). Polyunsaturated fatty acids (PUFA) are important nutrients in human diet because they limit the initiation of cardiovascular diseases, hypertension and arthritis (Echarte et al., 2001). Lipids in meat and meat products enhance flavor, aroma, juiciness and tenderness (Wen, 2013). The nutrients from meat are lost during aging and shelf life and the preservation methods define the available nutrients for human consumption. The post-mortem aging of meat results in flavor formation and protein break-down enhancing meat tenderness (Spanier et al., 1997; Khan et al., 2016). Traditionally, the carcass is hung on the rack to age without controlling the atmospheric temperature, airflow and humidity resulting in the loss of valuable nutrients (Ahnström et al., 2006). The most commonly available preservation methods include freezing, drying, salting and roasting (Arannilewa et al., 2005; Mohamed et al., 2019).

Thermal processing of meat has been reported to improve shelf life, food safety, flavor and taste. However, application of heat causes adverse physical and chemical changes affecting nutritional value, flavor and safety of food (Alizade et al., 2009). Triacylglycerols in fats and oils from meat and meat products, undergo oxidation reaction during frying, boiling and roasting in a process called lipid peroxidation producing primary oxidation products like hydroperoxides, epoxides, epidioxides and hydroxides (Zeb, 2012; Bauer-Plank and Steenhorst-Slikkerveer, 2000). The hydrolysis of hydroperoxide produces malondialdehydes (MDA), a secondary oxidation product whose concentration indicates the degree of lipid oxidation (Almroth et al., 2005). MDA causes off-flavor, changes in food taste (Mariutti and Bragagnolo, 2017; Purrinos et al., 2011) and initiates the development of cardiovascular diseases (atherosclerosis), cancer and is a mutagen for it reacts with DNA (Cline et al., 2004).

The quality of meat and meet products is compromised when frozen for a long period of time like losing color, undergoing lipid peroxidation and protein denaturation (Gonćalves and Junior, 2009). Oxidation of lipid and protein during freezing is associated with changes in flavor, texture and color which defines meat freshness 2017). Various authors have reported quality deterioration from frozen meat and meat products due to lipid peroxidation (Rey et al., 2001). Alterations in color, flavor and accumulations of carcinogenic primary oxidation products like hydroperoxides, radicals, aldehydes and epoxides has been reported during freezing of meet and meet products (Balev et al., 2005).

In frozen muscle tissues of fish and meat, bacteria and enzyme action results in the production of volatile bases like ammonia, trimethylamine, dimethylamine and other volatile acids (FAO, 1997). Proteins are broken down into ammonia whereas trimethylamine is a reduction product of trimethylamine oxide during meat spoilage under frozen shelf life (FAO, 1997). Total volatile basic nitrogen (TVB-N) has been used as an index for measuring meat freshness as ammonia, trimethylamine and dimethylamine content measurement (Vinci and Antonelli, 2002; Tecator, 2002).

Application of synthetic antioxidants, like butylated hydroxyanisole, butylated hyroxytoluene (BHT) and propyl gallate, has been used to limit the initiation of lipid peroxidation in meat and meat products (Yehye et al., 2015; Manessis et al., 2020). However, synthetic antioxidants have been linked to carcinogenicity and other health safety issues calling for the use of natural antioxidants to either scavenge peroxy radical chains or limit the formation of free radicals (Johnston et al., 2005). Despite the fact that synthetic antioxidants are safe when used with reference to relevant regulations like being applied as low as 0.02% w/w (Pokorný, 2007), meat industries have intensified research in natural antioxidants to meet consumers' perception of health safety (Shah et al., 2014). Natural antioxidants are extracted from plants with high concentrations of phytochemicals like phenolic compounds, ascorbic acids and carotenoids (Haque et al., 2020). The natural antioxidants have been extracted from leaves, roots, stems, seeds, fruits and barks of many plants like ginger, rosemary, garlic and oranges (Kim et al., 2019; Rojas and Brewer, 2008; Olatidoye et al., 2015).

Despite the global increase in the consumption of meat and meat products and the production of MDA in frozen meat limited studies have been conducted, in the majority of developing countries, including Malawi, on the application of natural antioxidants in frozen meat to limit the formation of MDA. Therefore, this study evaluated the effect of ginger, garlic and onion extracts on the quality of raw (chevon) goat meat and pork stored at the frozen temperature of -20°C for 14 days. In this respect, changes in quality of the frozen chevon and pork was determined by measuring 2-thiobartituric acid reactive substances (2-

*Corresponding author. E-mail: lecchatepa@yahoo.com, lchatepa@luanar.ac.mw.

TBARS) and TVB-N over a 14 day period

MATERIALS AND METHODS

Fresh thigh (chevon) goat meat and pork were purchased from the local butcheries at Mitundu and Nsabwe, local trading centers, in Lilongwe district. Fresh ginger, garlic and onion bulbs were purchased from Mitundu local market in Lilongwe district central Malawi.

Extraction and preparation of natural aqueous antioxidants extracts

The aqueous extract was prepared by following the method described by Majumdar et al. (2017) with minor modifications. Ginger, garlic and onion bulbs were ground in an electric food blender and 20 g of the ground materials were mixed with 100 ml of distilled water in a beaker for 24 h at room temperature. The crude aqueous extracts were filtered using a Whatmann filter paper and were evaporated to quarter volume at 50°C in the drying oven.

Sample preparation and chemical analyses

One hundred grams of steaks of chevon and pork were immersed in 100 ml of 10% (v/v) solution of the ginger, garlic and onion extracts for 24 h in beakers. After 24 h the samples were transferred into clean beakers and were frozen at -20°C for 24 h. The chemical analyses were conducted on days 0, 1, 3, 7, 14 and 21, respectively.

pH determination

The pH of the samples was measured by using a Kent pH meter in 10% (w/v) of the sample solution. 10 g of the sample was aseptically homogenised in 100 ml of distilled water, decanted and pH was measured (Olatidoye et al., 2015).

TVBN determination

Total Volatile Basic-Nitrogen was determined by using distillation method following the procedure described by Pearson (1976). 10 g of the sample was homogenised in 100 ml of distilled water in 250 ml quick fit flask, 1 g of magnesium oxide was added and the mixture was distilled for 15 minutes into 25 ml of 4% boric acid solution with 2 drops of mixed indicator. The distillate was titrated against 0.1M hydrochloric acid (HCl) (Pearson, 1976) and calculated as mg TVBN/g of sample as follows:

$$TVBN\ mg/g = (V_1\text{-}V_o) \times M \times 14.007/W$$

where V_1 and V_o are HCl titre volumes of sample and blank, respectively, M is the molar concentration of HCl.

TBARS value determination

TBARS content was determined with reference to the distillation method of Tarladgis et al. (1960) as described by Torres-Arreola et al. (2007) with minor modifications. Ten grams of the sample was minced into small pieces, transferred into a 250 ml quick fit flas and 47.5 and 2.5 ml of distilled water and 4 N HCl solution were respectively added. The mixture was swirled and distilled to collect 50 ml of the distillate. 5 ml of the distillate was pipetted into capped test tubes and 5 ml of 0.288% (w/v) solution of 2-thiobarbituric acid (TBA) in 50% (v/v) glacial acetic acid was added, the test tubes were capped and then boiled in a hot water bath for 35 minutes. Standard 1, 3, 3, 3 - tetramethoxypropane (TMP) samples of 0, 1, 2, 4, 8, 1.6 mg were prepared from a stock solution of 1 mg/ml by pipetting 0-6 ml into the test tubes. 5 ml of 2-TBA solution was added and the volume was made up to 10 ml with distilled water and was boiled for 35 min as the samples. The optical density was spectrophometrically measured at 532 nm using a UV-spectrophotometer. TBARS content as MDA, in mg/g, was calculated from the linear equation Y= 105742x as shown in Figure 1.

Statistical analysis

Laboratory chemical analyses were done in triplicate and the mean ± (SE) value of each chemical parameter was calculated using IBM SPSS version 20. The data was statistically analysed by using analysis of variance (ANOVA). T-test two-sample and unequal variances were used to compare mean values between treatments and meat type and significance was accepted at $P \leq 0.05$ level.

RESULTS AND DISCUSSION

pH changes of frozen chevon and pork with different treatments

Results on the pH changes of control and various treatment samples are presented in Figure 2. The initial pH values of control samples were 6.15 and 6.12 for chevon and pork, respectively. The various treatment samples pH values were 6.20, 5.48 and 6.40 and 6.76, 6.02 and 6.50 for raw/fresh chevon and pork mixed with 10% aqueous extracts of ginger, garlic and onion respectively. The pH of both control and treated samples increased after 3 days of frozen storage. However, the changes in pH slightly increased for both the controls and different treatments after 3 days of frozen storage which started decreasing after 7 days of storage. The lowest pH values of 5.63, 5.79 and 5.67 were observed in chevon compared to 6.67, 6.83 and 6.99 for pork mixed with 10% aqueous extracts of ginger, garlic and onion after 14 days of frozen storage, respectively.

The lowering/decreased pH values have been attributed to the presence of lactic acid bacteria in meat samples (Shin et al., 2017). Garlic and onions are reported to support the growth of lactic acid bacteria like *Lactobacillus*, *Weissella* and *Leuconostoc* species (Jung et al., 2012). The results from this study indicated that the lactic acid bacteria proliferated after 1 day of frozen storage which continuously kept the low pH of the samples up to day 14. The increase in the pH values after 1 day of storage could be attributed to protein degradation which results in amines production by micro-organisms in the meat (Biswas et al., 2004). It has been reported that the rise in pH of frozen stored meat is because of the production of volatile bases by endogenous or microbial enzymes. Alkaline ammonia like

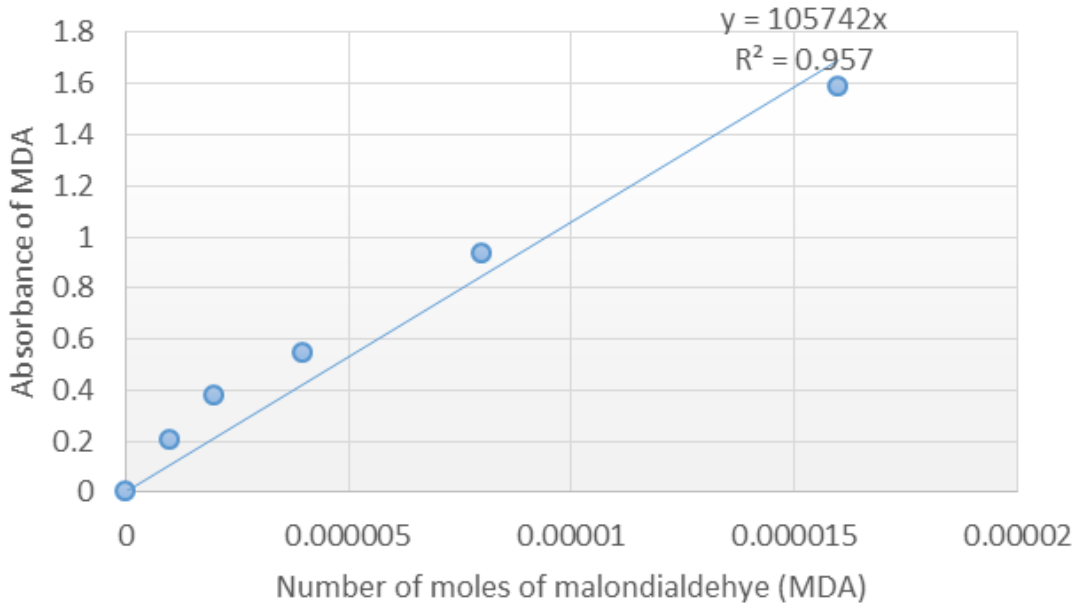

Figure 1. Standard curve of absorption of MDA against concentration.

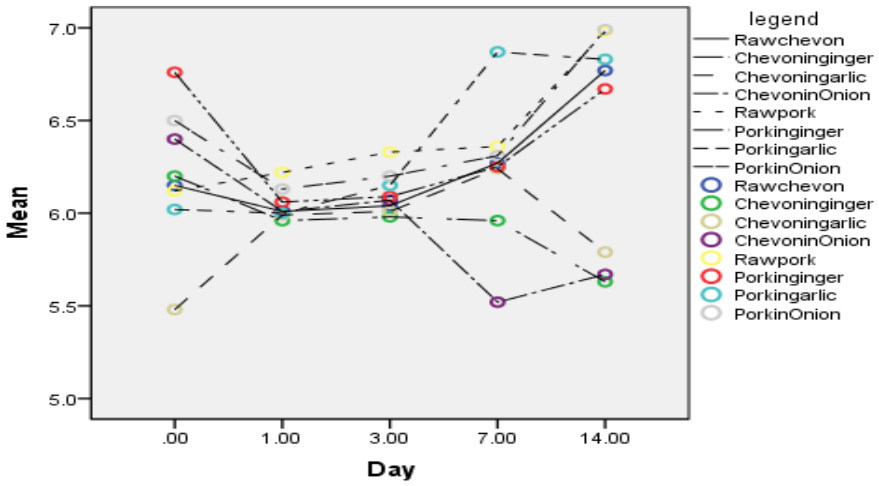

Figure 2. Changes in pH value of chevon and pork with different treatments during frozen storage at 20°C.

trimethylamine and ammonia are produced from amino acids after microbial decomposition (Masniyom et al., 2002). In this study, the observed low pH values could be attributed to the antioxidants present in the natural herbal spices which suppressed the growth of basic nitrogen metabolizing microbes in the meat (Cao et al., 2013).

2-TBARS content in frozen chevon and pork

Results on 2-TBARS as MDA content (mg/kg) for chevon and pork samples are shown in Table 1. Meat undergo lipid oxidation when the unsaturated fat and protein are exposed to molecular oxygen besides the processing conditions (Juntatoche et al., 2007) producing secondary oxidation products like aldehydes resulting in off-flavours in meat and meat products (Cao et al., 2013). In control chevon, TBARS content increased from 5.263 to 5.93 after 14 days of frozen storage. TBARS content in chevon treated with 10% aqueous extract of ginger, garlic and onion, decreased from 3.19±0.01, 4.01±0.01 and 4.47±0.00 to 2.03±0.01, 3.53±0.01 and 1.71±0.01 after 7 days of frozen storage but started increasing to day 14 of frozen storage. However, chevon mixed with 10%

Table 1. Mean composition of 2-thiobarbituric acid reactive substances (2-TBARS) [Mean ±SE] as MDA (mg/kg).

Day	Sample			
	Raw chevon	**Chevon ginger**	**Chevon garlic**	**Chevon Onion**
0	5.26±0.01^{a}	3.19±0.01^{aa}	4.01±0.01^{f}	4.47±0.00^{e}
1	4.84±0.2^{1b}	2.87±0.00^{bb}	3.29±0.00^{g}	2.42±0.02^{h}
3	2.06±0.01^{c}	2.76±0.02^{cc}	2.07±0.01^{i}	1.75±0.01^{j}
7	5.38±0.02^{d}	2.03±0.01^{dd}	3.53±0.01^{k}	1.71±0.01^{l}
14	5.93±0.01^{e}	4.71±0.03^{ee}	1.45±0.01^{m}	2.62±0.01^{n}
	Raw pork	**Pork ginger**	**Pork garlic**	**Pork Onion**
0	4.98±0.13^{b}	5.52±0.01^{bb}	3.69±0.01^{ff}	4.12±0.00^{x}
1	1.87±0.03^{a}	4.67±0.03^{aa}	6.59±0.04^{gg}	4.72±0.03^{hh}
3	2.00±0.01^{d}	1.47±0.01^{dd}	1.87±0.02^{ii}	3.51±0.01^{jj}
7	4.57±0.02^{c}	1.78±0.01^{cc}	2.03±0.01^{kk}	1.59±0.00^{ll}
14	3.92±0.01^{e}	2.26±0.01^{ee}	2.13±0.01^{mm}	3.50±0.20^{nn}

Mean ±SE within the same row and column with different superscript were significantly different at p≤0.05.

aqueous garlic extract had lower TBARS content of 1.45±0.01 followed by chevon treated with 10% aqueous onion extract (2.62±0.01) and chevon treated with 10% aqueous garlic (4.71±0.03) extracts at 14 day, respectively. In pork, similar trend as that of chevon samples was observed and TBARS values of 10% aqueous extract of ginger, garlic and onion decreased to 1.78±0.01, 2.03±0.01 and 1.59±0.00 with increasing days of frozen storage up to day 7. Pork in 10% aqueous garlic extract had the lowest TBARS value of 2.13±0.01 followed by pork in 10% aqueous onion extracts (3.50±0.20 mg/kg) and 10% aqueous ginger extracts (2.26±0.01), respectively. However, the final TBARS values after 14 days of frozen storage for pork treated with ginger, garlic and onion extracts were lower with the reported values of 2.26±0.01, 2.13±0.01 and 3.50±0.20 compared to 3.92±0.01 for control pork sample, respectively.

Cao et al. (2013) reported low TBA value of 1.60 mg /kg for stewed pork samples which increased to 4.81 mg/kg after 12 days of refrigeration. However, low values of 0.43±0.01 and 0.40±0.01 mg MDA/kg meat for old female and young goat were reported in India (Ahmad et al., 2016). Kim et al. (2019) reported decreasing TBARS values of 0.083±0.002 and 0.074±0.001 mg/kg meat for pork marinated with 3 and 6% ginger extracts compared to 9.5±0.2 mg/kg meat for the control pork samples. Similarly, 3 and 6% onion juices had 0.084±0.001 and 0.080±0.001 TBARS content compared to 0.095±0.002 for control pork samples (Kim et al., 2019). In pork patties mixed with fresh garlic, low TBARS values of 1.49 mg/kg has been reported and this value is lower compared to the value of 1.92 mg/kg for control pork patties reported by other authors (Kim et al., 2019).

The findings from this study have shown that TBARS values of 10% aqueous ginger, garlic and onion extracts mixed with chevon and pork samples were lower as compared to those of the control samples from day 0 to day 14 of frozen storage. This meant that antioxidants from ginger, garlic and onion were effective in reducing the extent of lipid oxidation in the frozen samples. Ginger, garlic and onion are reported to contain phenolic compounds, which are antioxidants, (El Diwani et al., 2009) which could limit lipid oxidation during frozen storage of the meat samples (Olatidoye et al., 2015). The high TBARS values in control chevon of 5.93±0.01 compared to 3.92±0.01 for pork, indicates that animal species is one of the factors that affect lipid peroxidation in the carcass shelf life (Min and Ahn, 2005). Lipid peroxidation is more susceptible in meat with high concentration of iron and myoglobin like raw beef and chevon compared to pork (Min et al., 2008; Estevez, 2015).

TVB-N content of frozen chevon and pork

The composition of TVB-N values, in mg/100 g, during frozen storage is presented in Table 2. TVB-N is a measure of the concentration of ammonia and primary, secondary and tertiary amines which defines the freshness of meat/muscles during storage (Fan et al., 2009). TVB-N values of chevon during frozen storage increased up to 3 days but decreased up to 14 days. However, the TVB-N values of treatment samples were less than those of control samples from 7 to 14 days. At 14 days, chevon mixed with 10% aqueous ginger extract had lower TVB-N value of 7.236±0.23 compared to 95.7±0.32, 12.367±0.23 and 13.77±0.23 for control and chevon mixed with 10% aqueous garlic and onion extract,

Table 2. Total volatile basic nitrogen (TVBN) [Mean ±SE] composition in mg/100 g.

Day	Sample			
	Raw chevon	**Chevon ginger**	**Chevon garlic**	**Chevon Onion**
0	46.58±1.42^{a}	12.71±0.34^{f}	12.76±0.29^{k}	11.68±0.47^{p}
1	234.24±5.27^{b}	14.40±1.40^{g}	31.27±3.03^{l}	13.30±2.10^{q}
3	98.00±4.04^{c}	106.41±2.91^{h}	126.01±4.04^{m}	86.34±1.68^{r}
7	43.94±0.59^{d}	17.37±0.76^{i}	35.00±0.81^{n}	54.60±0.81^{s}
14	95.70±0.32^{e}	7.24±0.23^{j}	12.37±0.23^{0}	16.61±0.50^{t}
	Raw pork	**Pork ginger**	**Pork garlic**	**Pork Onion**
0	10.72±0.26^{aa}	10.72±0.26^{ff}	10.72±0.26^{kk}	10.72±0.26^{pp}
1	4255.64±20.61^{bb}	89.97±4.67^{gg}	7.93±1.63^{ll}	27.53±2.26^{qq}
3	106.41±2.92^{cc}	35.94±3.27^{hh}	44.34±1.24^{mm}	33.14±1.24^{rr}
7	43.21±1.07^{dd}	42.49±0.48^{ii}	31.46±0.02^{nn}	65.80±0.81^{ss}
14	84.00±0.40^{ee}	14.23±0.62^{jj}	22.87±0.47^{00}	18.86±0.14^{tt}

Mean ±SE within the same row and column with different superscript were significantly different at p≤0.05.

respectively.

In pork, the TVB-N values of control and pork mixed with 10% aqueous ginger, garlic and onion extract were decreasing during the 14 days of frozen storage. At 14 days of frozen storage, pork mixed with 10% aqueous ginger extract had lower TVB-N value of 14.2341±0.617 compared to 84.004 ±0.4042 and 18.8581±0.1224 and 22.8678±0.4667 for pork mixed with 10% aqueous onion and ginger garlic and extracts, respectively. The TVB-N value obtained in this study of 22.87±0.47 for pork mixed with 10% aqueous garlic extract was lower compared to 25, 22.5 and15 mg/100 g meat for pork patties mixed 0.5% freeze-dried, freeze-dried fermented and freeze-dried aged garlic extract in a similar study conducted in Korea (Lee et al., 2019).

The increase in TVB-N values during storage of animal muscles is related to protein and amino acid breakdown by proteolytic Gram-negative bacteria and enzymes (Lefebvre et al., 1994). It is reported that the presence of microbes like lactic acid bacteria, *Brochothrix thermosphacta*, Enterobacteriaceae and *Pseudomonas* increases bio-chemical reactions in meat during storage resulting in high concentration of TVB-N (Tang and Yu, 2020).

However, *Allium* species like garlic and onion have high concentration of phenolic compounds, sulphur and phenolic compounds which have anti-fungal and anti-microbial properties (Benkeblia, 2005; Harris et al., 2001). The hydroxyl groups in *Allium* spp. reacts with either the sulfhydryl groups or some proteins of the bacteria limiting their proliferation in meat during storage (Harris et al., 2001). *Zingiber offinale* contains gingerol which has antimicrobial and antioxidant properties (Malu et al., 2009; Singh et al., 2017) and therefore has the ability of limiting microbial growth in meat during frozen storage thereby reducing both TVBN and TBARS values.

Conclusion

The findings from the present study have shown that aqueous extracts of ginger, garlic and onion have antioxidative properties which inhibit lipid peroxidation in fresh chevon and pork during frozen storage. Application of 10% aqueous extracts resulted in low pH values compared to the control samples. Chevon and pork samples treated with 10% aqueous ginger, garlic and onion extracts had low TBARS and TVB-N values compared to the fresh control samples. Therefore aqueous extracts from ginger, garlic and onions could be used to maintain quality and subsequently prolong shelf life of frozen chevon and pork to limit the development of TBARS and TVBN which initiates the development of cancer in human beings.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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