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African Journal of Biotechnology

Full Length Research Paper

Study of the effectiveness of essential oils of *Mentha aquatica* L. and *Pimpinella anisum* L. in the microbiological stabilization of pasteurized plant extract of *Cyperus esculentus* L. in Mali

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This study evaluates the antimicrobial properties of essential oils of *Mentha aquatica* and *Pimpinella anisum* in the stabilization of pasteurized plant extract of *Cyperus esculentus* in Mali. The extraction of essential oils was carried out by steam distillation of the aerial organs of *M. aquatica* harvested in Bamako and the seeds of *P. anisum* harvested at Diré in Timbuktu Region. Aging tests were carried out by direct incorporation of essential oils into plant extract of *C. esculentus* which is then pasteurized. The plants investigated have an essential oil extraction yield of 1.43% for *M. aquatica* and 2.14% for *P. anisum.* The results of the aging tests showed that the essential oils of *M. aquatica* and *P. anisum* exert a strong antimicrobial activity on the spoilage flora because it made it possible to extend the storage time of vegetable extract up to 32 days. The oils of these plants are therefore an effective alternative to chemical antimicrobials, many of which are harmful to the health of the consumer and it would be interesting to study the biological effect of the combination of these essential oils in the stabilization of this food since they are active on different microbial groups.

Key words: Pasteurized plant extract of *Cyperus esculentus*, essential oils, antimicrobial activities, preservation.

INTRODUCTION

In food technology, controlling the microbiological quality of food is one of the essential bases of its ability to satisfy consumer health safety. The exposure of food to microbial spoilage following the lack of control of this parameter leads to a reduction in its sensory, nutritional, and health characteristics (Bolnot et al., 1985) and its market value. Similarly, losses linked to the production of allergenic compounds and mycotoxins by molds, which are also responsible for the formation of unpleasant tastes in food, are frequently recorded (Ownagh et al., 2010).

Preservation technology, dealing with many problems

of oxidation and microbial contamination of foodstuffs, has called on chemistry which has allowed the appearance and use of additives as synthetic food preservatives. These chemicals, used to prevent food spoilage (Nakahara et al., 2003) have proven to be sources of several harms to consumers. Indeed, their application in different industrial sectors (in particular agri-food, insecticide, perfumery, cosmetics, and pharmaceuticals) could be the cause of toxic, carcinogenic, or even mutagenic effects on health; the more reason for consumers to be tempted by a chemical-free diet. This is pushing manufacturers to carry out discussions aimed at reducing the use of additives resulting from chemical synthesis and to develop research and the use of natural products as preservatives with antimicrobial and antioxidant activities in foodstuffs.

Traditionally, empirical knowledge has enabled different civilizations to use plants, particularly aromatic plants, as food additives used as a seasoning or to increase the shelf life of foods (Cheng et al., 2013). Essential oils and aromas constitute in this context the major part of the natural compounds extracted from aromatic plants. These essential oils are now arousing more and more interest for industrialists and researchers because of their strong antibacterial, antifungal, and antioxidant activities (Dung et al., 2008) making them more interesting as natural preservatives in food industries (Gachkar et al., 2007; Rasooli et al., 2008).

It is with this concept in mind that the essential oils of *Mentha aquatica* L. and *Pimpinella anisum* L. belonging, respectively to the Lamiaceae and Apiaceae families were tested in the preservation of a very perishable food, namely the plant extract of *Cyperus esculentus*. These botanical families have been the subject of several investigations by researchers. Also, a search in the scientific literature indicates that there are few reports of scientific studies, particularly on the prospects for extending the shelf life of plant extract of *C. esculentus* in Mali. The plant extract of *C. esculentus* is a milky drink extracted from its tubers after turgidity and grinding. The very sweet *C. esculentus* tubers, called “tigernut” in English, “chufa” in Spanish, “Souchet ou pois sucré” in French, and “tchôgôn” in Bamanankan, are rich in carbohydrates (49%, mainly starch (25%)), lipids (25%), vitamins (C and E), minerals (Ca, Fe, K, P) and crude fiber (Coskuner et al., 2002; Arafat et al., 2009; Ukwuru and Ibeneme, 2011). In Mali, *C. esculentus* is mainly grown in the Sikasso region, which is the main supplier of tiger nut tubers to the Malian populations, who generally use them as snack food.

However, tiger nut tubers are used in Spain for the production of a very attractive milky drink called “horchata de chufa” (Arafat et al., 2009; Ukwuru and Ibeneme, 2011); whose production and marketing are limited by two major constraints:

(1) Its high microbial load (107 CFU/mL) which requires its consumption within 24 h of its production (Aliyu et al., 2022);
(2) The high proportion of starch in this drink makes it difficult to heat treat at the risk of gelatinization of this polysaccharide.

It is in this context that this study takes place to evaluate the possibility of extending the shelf life of pasteurized tiger nut extract using natural food additives such as essential oils.

MATERIALS AND METHODS

Collection of plant material and extraction of essential oils

Essential oils were extracted from the aerial organs of *M. aquatica* and the dried seeds of *Pimpinella*. The aerial parts collected in Bamako were dried in the shade, while the dried anise seeds came to us from Diré in the Timbuktu region. The extraction of essential oils, which lasted 4 h, was carried out by steam distillation using a modified Kaiser-Lang steam circulation device. The essential oils are then dehydrated by crystallization of the residual water in the freezer and stored at +4°C. The extraction yield (Y) is expressed according to the following formula:

$$Yield\ (\%) = \frac{Weight\ (g)\ of\ essential\ oil}{\text{Weight of dry plant material}}$$

Production of pasteurized tiger nut extract

The pasteurized tiger nut extract used as a test food was produced at the quality control laboratory of the University of Ségou from tubers coming directly from a field located in the village of Ifola in the Sikasso region of Mali. The samples are stored under cold conditions at +2°C.

Aging test

The tiger nut extract aging tests were carried out by direct incorporation of each essential oil into 40 mL of tiger nut extract at varying doses. Thus, taking into account the organoleptic parameters (flavor, taste, smell) of the tiger nut extract and essential oil mixture, three doses were tested during the experiment: 10, 20 and 30 µL/40 mL. A control (without essential oil) was also tested. After homogenization by manual rotation, the samples were put under observation and periodic samples of four days followed by analyses were carried out to follow the evolution

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of the microbiological quality of the vegetable extract put in conservation.

Monitoring the evolution of the microbiological quality of plant extract of C. esculentus during storage

A cascade dilution is carried out from the mixture formed by the tiger nut juice and the essential oil. For each quantity of essential oil used, two tubes were used for the decimal dilution in addition to the control. Indeed, in two sterile tubes were introduced 9 mL of physiological water. 1 mL of the product constituting the stock solution or 10° is added to one of the tubes containing 9 mL of physiological saline, this is the 10^{-1} dilution. From this well-stirred mixture, 1 mL is taken, which is then added to 9 mL of physiological water contained in the next tube. This solution corresponds to the 10^{-2} dilution. Microbiological quality parameters were assessed using standard microbiological analysis methods. The germs sought consisted of total aerobic mesophilic flora (total germs; NF V08-051) and fungal flora (ISO 7954). The culture media chosen in this study consisted of Plate Count Agar (PCA) for the total flora and SABOURAUD for the fungal flora.

Statistical analyses

The data obtained was entered in the Excel 2017 spreadsheet and then analyzed by the R software. These data are analyzed by the ANOVA method to determine the differences. For this purpose, the differences are significant if $p < 0.05$; they are not significant if $p > 0.05$.

RESULTS

The plants were investigated to have an essential oil extraction yield of 1.43% for *M. aquatica L.* and 2.14% for *P. anisum*. Tables 1 and 2 present the evolution of the total bacterial flora (TBF) and the fungal flora (FF) in the plant extract of *C. esculentus* supplemented, respectively with essential oil of *M. aquatica* and of *P. anisum* with a view to extend its shelf life. These results show that the antimicrobial activity of essential oils depends on the nature of the essential oil, the dose and even the group of microorganisms targeted.

Indeed, with the essential oil of *M. aquatica* L., Table 1 shows us a slight slowing down of bacterial growth in the samples containing the 10 and 20 µL doses until the 12th day of storage. While at the level of the sample containing the dose of 30 µL of essential oil of *M. aquatica*, the reduction of the TBF continues until the 20th day of storage of the plant extract of *C. esculentus*. In addition, a gradual and total destruction of the fungal flora is observed throughout the storage period, at all doses of the essential oil. This result shows the effectiveness of the essential oil of *M. aquatica* in controlling the bacterial load and in inhibiting the fungal flora contaminating the plant extract of *C. esculentus*. There is also a start of tiger nut extract fermentation on the 40th day of storage in the sample containing the 20 µL dose and the control. While those containing the 10 and 20 µL doses are fermented on the 44th day of storage. This result shows that in terms of stabilizing the tiger nut extract produced in this study, the 10 and 20 µL doses are more effective than the 30 µL one. Moreover, the multivariate analysis reveals that the doses applied to the samples have statistically identical effects ($p > 0.05$). However, we can also see that the 30 µL doses of the essential oil significantly reduce fungal growth compared to the lower doses. This essential oil therefore shows an effective fungicidal effect and a bacteriostatic effect, respectively against fungi and bacteria contaminating tiger nut extract.

As for the essential oil of *P. anisum* in Table 2, a slowdown was also noticed in the growth of the bacterial flora until the 8th day of storage with the dose of 10 µL, until the 12th day with the dose of 20 µL and until the 16th day with the dose of 30 µL beyond which the bacterial growth resumes but timidly. There is also a reduction in fungal growth up to the total destruction of FF in the tiger nut extract studied at all the doses tested. This result therefore shows the effectiveness of the essential oil of *P. anisum* in the fight against fungi contaminating the plant extract of *C. esculentus*. A start of fermentation can also be observed from the 36th day of storage in the samples containing 10 and 20 µL of essential oil and the control. The last fermented sample on the 40th day of storage is the one containing 30 µL of essential oil. This result shows that among the doses tested, the 30 µL dose is more effective in stabilizing tiger nut extract compared to the lower doses tested. The analysis of variance of these results showed that the differences observed are non-significant ($p > 0.05$) with regard to the counted fungal load, but significant at the level of TBF ($p < 0.05$). In addition, the averages of the colonies counted are lower in the samples containing the essential oil of anise than those not containing it. This means that the added doses of essential oil reduced the fungal load in the pasteurized extract samples. It should also be noted that a decrease in this microbial load is all the more important as the quantity of essential oil increases. This result therefore shows the effectiveness of the essential oil of *P. anisum* in the fight against fungi contaminating the tested pasteurized extract plant of *C. esculentus*.

DISCUSSION

The yields obtained following the extraction of essential oils from the different plants studied showed differences compared to the bibliography. Indeed, the extraction yield of the essential oil of *M. aquatica* is higher than those of the work of Dai et al. (2015) in Cameroon and of Morteza-Semnani et al. (2014) in Iran, and lower to that

Table 1. Effect of essential oil of *Mentha aquatica* L. *1753* on the evolution of total bacterial flora (TBF) and fungal flora (FF) in *Cyperus esculentus* nut extract.

Sampling	TBF (witness)	TBF (10)	TBF (20)	TBF (30)	FF (witness)	FF (10)	FF (20)	FF (30)
Day0	190	210	180	150	140	130	120	110
D+4	210	180	150	110	140	110	100	80
D+8	230	150	110	80	160	90	70	50
D+12	270	160	80	50	190	50	40	40
D+16	340	230	120	30	220	30	20	20
D+20	520	470	180	20	260	20	10	10
D+24	700	580	270	60	270	10	0	0
D+28	980	610	310	100	310	10	0	0
D+32	1450	1150	510	180	390	0	0	0
D+36	5600	1300	750	300	560	0	0	0
D+40	Fermented product	1800	900	Fermented product	Fermented product	0	0	Fermented product
D+44	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product

Table 2. Effect of essential oil of *Pimpinella anisum L.1753* on the evolution of total bacterial flora (TBF) and fungal flora (FF) in *Cyperus esculentus* nut extract.

Sampling	TBF (witness)	TBF (10)	TBF (20)	TBF (30)	FF (witness)	FF (10)	FF (20)	FF (30)
Day0	10	40	40	40	10	30	20	60
D+4	10	30	30	40	10	20	10	30
D+8	30	20	20	30	10	10	10	10
D+12	60	40	10	10	10	10	0	0
D+16	70	80	20	10	10	0	0	0
D+20	110	180	30	70	10	0	0	0
D+24	130	200	50	110	10	0	0	0
D+28	140	800	910	200	10	0	0	0
D+32	230	2900	2100	380	20	0	0	0
D+36	Fermented product	Fermented product	Fermented product	1100	Fermented product	Fermented product	Fermented product	0
D+40	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product	Fermented product

of Pavela et al. (2014) in the Czech Republic. That of the essential oil obtained from the seeds of *P. anisum* is lower than those obtained by Ullah et al. (2015) in Pakistan, Saibi et al. (2012) in Algeria and Arslan et al. (2004) in Turkey.

However, the differences observed could be affected by several factors, namely the harvest period, the stage of development of the plant, the nature of the soil, the geography, the cultural practices or even the extraction techniques (Marzouki, 2009; Olle and Bender, 2010).

In addition, this study focused on the evaluation of the antimicrobial activities of essential oils of two (2) aromatic plants in the stabilization of pasteurized plant extract of *C. esculentus L.* in

Mali. The results obtained confirm their antimicrobial properties and the importance of their use in the conservation of plant extract of *C. esculentus*. Indeed, the bacterial and fungal flora studied have shown themselves to be sensitive to the antimicrobial actions of the essential oils tested. Thus, Tables 1 and 2 allow us to better understand the behavior of microbes in the plant extract of *C. esculentus* studied and to estimate its storage time. The shelf life obtained with the essential oils tested explains their significant interest in being used as a preservative in the food industry. This result confirms the data of Degnon et al. (2016) which stipulate that the use of the essential oil of *M. piperita* is possible in the agri-food industry with a view to stabilizing cow's milk in southern Benin. While maintaining the microbial load below the Spanish standard which is 2.5×10^5 CFU/mL (Mosquera et al., 1996), the essential oils of these aromatic plants of the Lamiaceae and Apiaceae families have demonstrated their ability to delay the process of oxidation in the tiger nut extract tested. These results confirm the work of Getahun et al. (2008) and Barakat et al. (2016) and who have all demonstrated the antimicrobial and antioxidant powers of the essential oils of these species.

It should be noted that all of the results obtained are superior to those obtained by Selma-royo et al. (2022) who estimated the shelf life of tiger nut extract at +2°C for up to 3 weeks if it is processed at a temperature of 70-75°C for 40-60 s. Also, the maintenance of all the essential oils of the microbial load below the Spanish standard which is 2.5×10^5 CFU/mL confirms their ability to retard microbial growth in tiger nut extract. All of these results confirm not only the antimicrobial activity of essential oils but also their ability to delay the oxidation of foodstuffs. They therefore indicate that the industrial use of these essential oils could be envisaged with a view to stabilizing tiger nut extract. This is also the case with other products in the agri-food sector where essential oils are used in many sectors, particularly in bakery and cheese (Vazquez et al., 2001), in charcuterie (Quintavalla and Vicini, 2002) and in confectionery (Lanciotti et al., 2004). This trend is increasingly motivated by the attraction of industrial companies towards green consumption, which are increasingly resistant to synthetic additives because of the many harmful effects associated with the use of these antimicrobials and food preservatives chemical synthesis (Burt, 2004). Similarly, Degnon et al. (2016) state that essential oils possess the advantage of being bioactive in the vapor phase, a characteristic that makes them useful as possible fumigants for the protection of stored products.

Also, spices and plant extracts including essential oils, known since antiquity as having many virtues (antibacterial, antifungal and antioxidant) are increasingly used in food preservation (Bounatirou et al., 2007) due to their relatively safe status, wide consumer acceptance and exploitation for potentially versatile functional use (Lan-phi et al., 2009).

Conclusion

This study made it possible to evaluate the antimicrobial activities of essential oils of seven aromatic plants tested in the microbiological stabilization of pasteurized plant extract of *C. esculentus L.* in Mali. In view of the results obtained, we can conclude that these plants could constitute an effective alternative to replace synthetic antimicrobials, several of which have been harmful to the health of the consumer and to the environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Optimization of regeneration protocol and prospecting spectinomycin resistance in barley (*Hordeum vulgare* L.) cv Haider-93

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Immature zygotic embryos from spring barley cv. Haider-93 were used to induce somatic embryogenesis. The type of the explant, the level of 2,4-dichlorophenoxyacetic acid (2,4-D) and handling of calli during subculture are critical factors to obtain maximum number of regenerants. Different concentrations of 2,4-D (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L) and Kinetin (0.5, 1.0, 1.5, 2.0 mg/L) were used for callus induction and shoot initiation, respectively. Use of immature embryos having damaged axis as explants revealed a pronounced gradient of callus formation on Murashige and Skoog (MS) medium supplemented with 2.5 mg/L of 2,4-D and maximum regeneration response at 1 mg/L kinetin. Regenerated shoots were rooted on MS medium supplemented with 1 mg/L IAA. A kill curve was developed to find out the sensitivity level of barley cells to spectinomycin, a broad-spectrum antibiotic. This study fulfils an antique prerequisite of a reproducible regeneration system required for the improvement of barley via genetic engineering and also falls under, "Establish Good Health and Well-Being" Sustainable Development Goals of United Nations Organization.

Key words: 2,4-Dichlorophenoxyacetic acid, indole acetic acid, kinetin, antibiotic resistance, spectinomycin, Barley 53.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world after wheat, maize and rice (Rostami et al., 2013). Barley is a self-pollinated crop belonging to family Poaceae and genus *Hordeum* (Hu et al., 1983) which evolved from *Hordeum spontaneum* (Nevo, 1992). Genus *Hordeum* comprises 25 to 30 species distributed all around the world.

Barley is a diploid species with two sets of chromosomes (2n=2x=14) and base number is seven. Barley is an annual, short duration crop and additionally

have good adoptability, that is, it can be cultivated from 42° latitude South to 70° latitude North. Seed is an edible part of barley plant, formed in the palea of spikelet. Seeds of barley are harder than any other cereal. Barley can be cultivated in all extreme climates including arctic, subarctic and dry areas (Guo, 1987; Baik and Ullrich, 2008), but high humidity affects its growth (Smith, 2004). Barley grain is mostly used in malt production and animal feed because of its high nutritional value which is 95% more than that of maize. Malt from barley is used in beer making, analytical grade alcohol production, whisky, and syrups. About 1000 million people in Tibetan cuisine and Medieval Europe use barley as staple food. Mostly 85% cultivated barley is used as animal feed while the remaining is utilized in malting and brewing industry for human consumption.

Barley has high nutritional value possessing maximum value of vitamins, minerals, fiber and bioactive compounds namely β-glucans, tocopherols, tocotrienols and phenolic compounds that are an excellent source of antioxidants for disease prevention (Baik and Ullrich, 2008; Gallegos-Infante et al., 2010). High content of soluble fibers found in Barley assist in the prevention of constipation and colon cancer whereas insoluble fibers aid in lowering blood cholesterol leading to the prevention of cardiovascular diseases (Ötles and Ozgoz, 2014). Barley has high content of β-glucans than any other cereal (Zhang et al., 2003), containing numerous vitamins like thiamine (vitamin B1), niacin (vitamin B3), and minerals such as iron, copper, magnesium, phosphorus, zinc, and selenium (Pins and Kaur, 2006). Barley can store 15% proteins (by dry weight) for up to 10 years (Tanasienko et al., 2011). In some areas like California, barley is used for hay production.

There are some oriental countries, like Korea, Japan, China, and Himalayan using naked barley for cooking, grinding, and beverages production.

Cultivation area of barley is declining day by day due to urbanization and different stresses such as biotic and abiotic stress. The only possible way to improve yield of barley is by producing new varieties with high resistance against biotic (El-Sappah et al., 2021a) and abiotic stress factors by employing robust genetic engineering and gene silencing techniques such as CRISPR/Cas9 and RNAi (Abbas et al., 2020; El-Sappah et al., 2021b). An *in vitro* reproducible regeneration system including dedifferentiation, redifferentiation, regeneration, and organogenesis is the prerequisite for genetic transformation against biotic and abiotic stresses. Tissue culture is also helpful in seed cost reduction, better crop improvement and seed importation. Development of tissue culture and regeneration protocol is vital for application of biotechnological tools for genetic improvement, soma clonal variants recovery, transgenic production, clonal propagation, pathogen free plants production and preservation of germplasm (Ehsanpour and Jones, 2000; Ganeshan et al., 2003). Antibiotic resistance genes are antique part of vectors for identification of successful transgenic events. Antibiotic along with Green Fluorescent Protein (GFP) have proved most suitable selection system (Khan and Maliga, 1999). Noticeably, some plants naturally harbor resistance against antibiotics. Therefore, it is mandatory to undermine lethal dose of a broad-spectrum antibiotic in barley.

In this study, immature embryos of barley were cultured on medium supplemented with different concentrations of hormones for callus induction, proliferation and regeneration. Barley seeds were also cultured on MS medium supplemented with different concentrations of broad spectrum antibiotic spectinomycin to prospect resistance inheritance of barley. Spectinomycin is used as conditional positive selectable marker for selection of transgenics (Mustafa and Khan, 2012) which causes bleaching by inhibiting chlorophyll biosynthesis (Oreifig et al., 2004). The present study was conducted to provide precise supportive bench for genetic transformation for the improvement of cereal crop barley against biotic and abiotic stress and increasing production.

MATERIALS AND METHODS

Explant for callus induction

Seeds of barley (*H. vulgare* L.), commercial cultivar Haider-93 were collected from Ayub Agricultural Research Institute (ARRI), Faisalabad. Seeds were cultivated by sowing in pots filled with mixture of sand, silt and organic matter at a ratio of 2:2:1 and placed in green house. At flowering, spikes were covered with brown bags to avoid cross pollination and immature seeds (DAP=15) were used for callus induction and regeneration. Immature seeds were surface sterilized with 70% (v/v) ethanol for 1 min, and then with 3% sodium hypochlorite (NaOCl) for20 min and finally five washings with sterile double distilled deionized H_2O were given (Salama et al., 2013; Abbas et al., 2016). Immature embryos were dissected and cultured in Petri plates containing MS medium supplemented with different concentrations of 2,4-D. Cultures were maintained at 26±2°C in light under a 16 h photoperiod.

Medium for callus induction

The basal medium for callogenesis, regeneration and root formation was MS medium (Murashige and Skoog, 1962). Stock

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Table 1. Different concentrations of 2,4-D in culture medium used for callus induction.

S/N	Treatments	Media compositions
1	T_0	MS0
2	T_1	MS + 0.5 mg/l 2,4-D
3	T_2	MS + 1.0 mg/l 2,4-D
4	T_3	MS + 1.5 mg/l 2,4-D
5	T_4	MS + 2.0 mg/l 2,4-D
6	T_5	MS + 2.5 mg/l 2,4-D
7	T_6	MS + 3.0 mg/l 2,4-D

Table 2. Plant regeneration medium for immature embryos derived calli of cv. Haider-93.

S/N	Treatment	Media composition
1	T_0	MS0
2	T_1	MS + 0.5 mg/L kinetin
3	T_2	MS + 1.0 mg/L kinetin
4	T_3	MS + 1.5 mg/L kinetin
5	T_4	MS + 2.0 mg/L kinetin

solutions of MS salts, vitamins and growth hormones were formed in double distilled deionized H_2O. As a source of C, sucrose was added in medium at a concentration of 30 g/L. pH of culture medium was adjusted to 5.8 and solidified with 8 g/L agar. Medium was autoclaved at 121°C at 15 psi for 20 min, aliquots (30 ml) of each medium were poured in the Petri plates and wrapped with clink film and incubated at room temperature to examine contamination (Cheng et al., 2021). MS plane medium supplemented with different concentrations of spectinomycin was used to examine resistance inheritance of barley.

Immature embryos were excised under aseptic conditions in laminar flow hood with the help of scalpel; embryogenic axis were damaged with the help of sterilized surgical blade and cultured on MS medium (Murashige and Skoog, 1962) supplemented with various concentrations of 2,4-D to optimize callus induction (Table 1). Plates containing cultured embryos were incubated at 23±2°C under dark condition. Calli were sub-cultured on the same medium after a two-week interval for proper nutrient supply. For the determination of callus growth rate, calli were transferred to a sterile Petri dish and the weight of calli present in a plate was measured in aseptic conditions. Afterwards, callus pieces were re-transferred to callus culture medium. This measurement was repeated every week for 8 weeks.

Medium for regeneration

Half strength MS medium supplemented with different concentrations of kinetin (0.5, 1.0, 1.5, 2.0 mg/L) (Table 2) was used for regeneration of plantlets by incubating callus in growth room at 25±2°C, 16 h light and 8 h dark (Abbas et al., 2020). After dark incubation period, calli were transferred onto Petri dishes containing regeneration medium (MS) supplemented with kinetin for shooting. After 4-, 8- and 12-weeks data was collected as the number of shoots regenerated per explants. For shoot regenerating explants, numbers of shoots per each explant were also recorded (Ahloowalia, 1982).

Rooting of *in vitro* regenerated shoots

Shoots of specific height (1.5 cm) were removed from medium containing kinetin for regeneration and transferred in 400 ml Magenta boxes containing 50 ml of half-strength MS medium supplemented with 1.0 mg/L IAA for rooting. Magenta boxes were incubated at 25±2°C in growth room. Regenerated plants were removed from the magenta boxes after they reached the expected developmental stage. The roots were then completely cleaned with distilled water after being properly rinsed with tap water to remove the media.

Plants were transferred in pots containing sterile clayey soil and sand (50:50), covered with polythene bags and placed in growth chamber at 21±1°C and 13% relative humidity. After 15 days of acclimation, plants were shifted in greenhouse and fertilized with NPK fertilizers mixture with a ratio of 17:17:17 (Aguado-Santacruz et al., 2011).

Prospecting antibiotic resistance

An effective antibiotic with its appropriate concentration is requisite for identification of successful transformation system (Mustafa and Khan, 2012). After sterilization, barley seeds were transferred onto MS0 medium (Murashige and Skoog, 1962) with various concentration of antibiotic (Table 7) to examine the resistance inheritance of barley (*H. vulgare* L). Magenta boxes were visited regularly to monitor the effect of antibiotic on the germinated plantlets of barley.

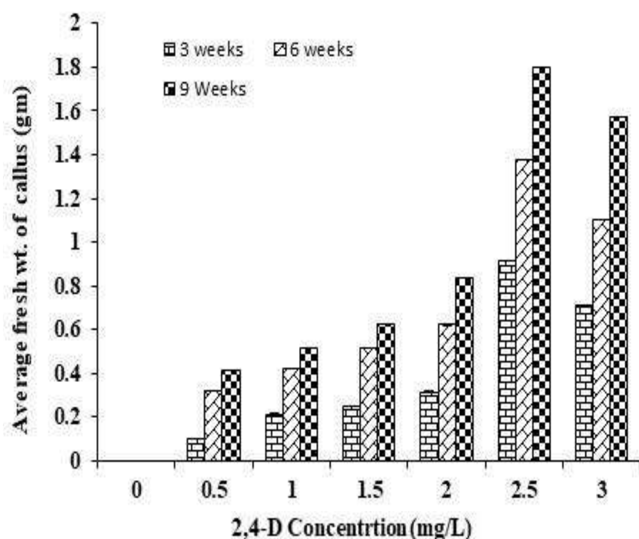

Figure 1. Different concentrations of 2,4-D for callus induction in immature embryos of barley.

RESULTS

Callus induction on MS + auxin (2,4-Dichlorophenoxy acetic acid)

Efficient and reproducible *in vitro* regeneration techniques leading to the production of plants from cultured tissues are of prime importance to clonal propagation and other genetic engineering approaches of barley plants. Callogenesis from immature embryos (DAP=15) of barley (*H. vulgare* L.) cultured on MS medium supplemented with different concentrations of 2,4-D was initiated within 3 to 4 days (Figure S1) of culture and maximum callus induction frequency (95%) was achieved on MS medium supplemented with 2,4-D at the concentration of 2.5 mg/L (Figure 1). Further increase in 2,4-D negatively affected callus induction and its weight (Table 3). For three weeks after incubation, callus was observed for proliferation purpose to get appropriate mass. We observed shoots initiation in some calluses which were removed with the help of scalpel in aseptic conditions (Figure S2). After three weeks of incubation, efficient callus induction frequency and size was calculated by applying the following formula.

Callus induction (%) = No. of embryos product calli / Total No. of embryos cultured × 100

The callus mass and frequency were recorded after every three weeks of inoculation for a period of 9 weeks, at maximum. Callus induction, proliferation frequency and its weight varied with respect to the concentration of 2,4-D used (Table 3).

Morphological characterization of callus

Calli induced in immature embryos were characterized for their amount, color and texture induced at various concentrations of 2,4-D (Table 4). After 8 to 9 weeks of incubation, friable calli were developed which were characterized morphologically (Figure 2). No callus induction (-) was observed on MS plane medium; poor (+) amount, creamy, whitish, creamy white, compact and watery callus were induced on MS medium supplemented with 0.5, 1.0 and 1.5 mg/L of 2,4-D, respectively. Good (+ + +) amount, creamy white and friable callus were induced on MS medium supplemented with 2.0 mg/L of 2,4-D, while best (+ + + +) and highest amount of callus with creamy white color and friable texture were induced on MS medium supplemented with 2.5 mg/L of 2,4-D. Finally, satisfactory (+ +) amount with creamy white color but watery callus were induced on MS medium supplemented with 3.0 mg/L of 2,4-D (Table 4).

Callus to plant regeneration on MS + kinetin medium

Calli were observed for length and number of shoots initiation after 4, 8 and 12 days. The lengths and numbers of newly regenerated shoots from different calluses cultured for shoot initiation on MS medium supplemented with the aforementioned concentrations of kinetin were varied (Figure S3). Within 4 days, shooting primordial became visible and after 12 days, maximum number of shoots was regenerated from calluses (7-11 shoots/ callus) with lush green color (Figure 3 and Table 5). Maximum numbers of shoots (11) with lush green color and appropriate length (1.4 cm) were observed on MS medium containing 1.0 mg/L of kinetin (Table 5). Highest shoot regeneration efficiency was measured on MS medium supplemented with 1.0 mg/L of kinetin calculated by applying the following formula (Table 5).

Embryogenic efficiency (%) = No. of shoots producing calli / No. of embryos incubated × 100

Rooting and acclimation of regenerated shoots

Cluster of roots were initiated within 4 days after transferring regenerated shoots on rooting medium and significant number of roots were developed within 12 days (Figure 4). Maximum rooting frequency was observed on MS medium supplemented with 1.0 mg/L IAA calculated by the following formula (Table 6).

Regeneration efficiency (%) = No. of root producing calli / No. of incubated embryos × 100

Plants with developed roots were shifted to half liter pots

Table 3. Different concentrations of 2,4-D for callus induction in immature embryos of barley (*Hordeum vulgare* L).

Concentration of 2,4-D (mg/L)	Average fresh weight (g)			Total cultured embryos	Callus induction frequency (%)
	3 weeks	6 weeks	9 weeks		
0.0	Control	Control	Control	90	0
0.5	0.101 ± 0.001	0.321 ± 0.0005	0.414 ± 0.0005	90	2
1.0	0.210 ± 0.005	0.422 ± 0.0005	0.513 ± 0.0005	90	9
1.5	0.251 ± 0.0005	0.513 ± 0.0005	0.622 ± 0.0005	90	22
2.0	0.315 ± 0.0005	0.621 ± 0.0005	0.837 ± 0.0005	90	58
2.5	0.914 ± 0.0005	1.374 ± 0.0005	1.798 ± 0.0005	90	95
3.0	0.712 ± 0.0005	1.103 ± 0.0005	1.571 ± 0.0005	90	44

Table 4. Morphological characterization of callus of barley cv. Haider-93.

Treatment (MS+2,4-D)	Callus amount	Callus morphology/Explant callus color	Callus texture
0	-	-	-
0.5	+	Creamy	Compact
1.0	+	Whitish	Compact
1.5	+	Creamy White	Watery
2.0	+ + +	Creamy White	Friable
2.5	+ + + +	Creamy White	Friable
3.0	+ +	Creamy White	Watery

(-) No callus; (+) Poor callus; (+ +) Satisfactory Callus; (+ + +) Good Callus; (+ + + +) Best Callus.

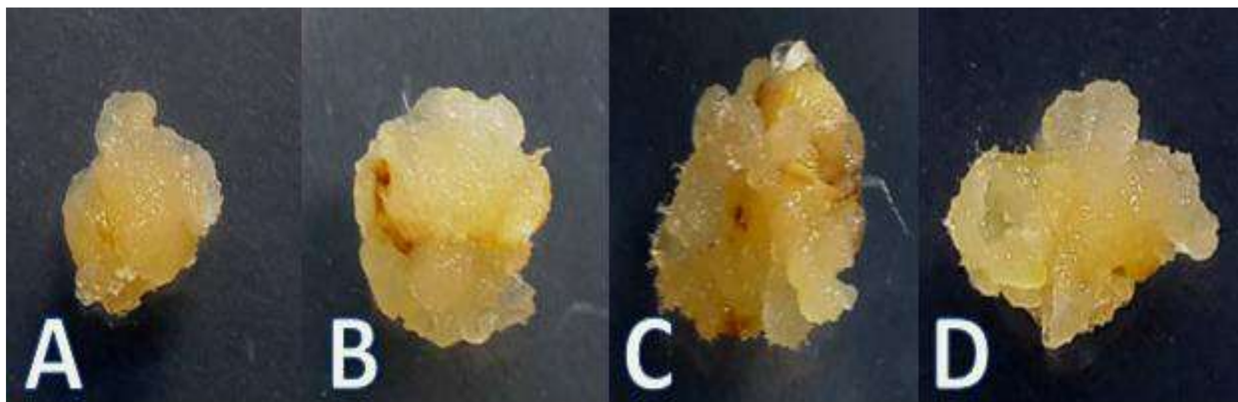

Figure 2. Callus formed on MS medium supplemented with 2.5 mg/L 2,4-D (A) after 3 weeks, (B) 6 weeks, (C) 7 weeks, and (D) 9 weeks.

filled with soil and sand (50:50) along with organic matter, covered with transparent polyethylene bags and placed in a growth chamber at 21±1°C, at a 13% relative humidity and at a photon flux of 70 µmol m^{-2} s^{-1} for hardening.

After 2 weeks, pots were shifted to room temperature and regularly fertilized with NPK (17:17:17) (Peng et al., 2019).

Prospecting spectinomycin resistance in barley

Change in color or chlorosis of leaves was observed indicating spectinomycin had not affected pigmentation in chlorophyll and barley exhibited strong resistance. Although root and shoot length varied on medium supplemented with different levels of spectinomycin, all

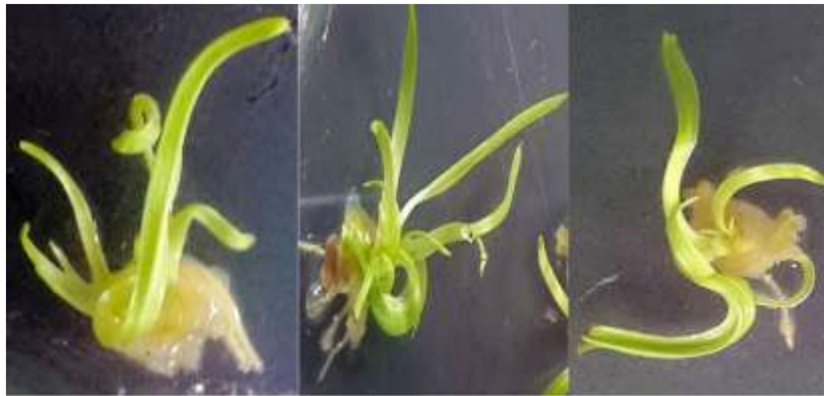

Figure 3. Regenerated plantlets from callus induced in immature embryos of barley (*Hordeum vulgare* L).

Table 5. Effect of kinetin on regeneration of calli of cv. Haider-93

S/N	Conc. of kinetin (mg/L)	No. of shoots			Shooting efficiency (%)
		4 days	8 days	12 days	
1	0.5	2 ± 0.57	5 ± 1.73	7 ± 1.15	67
2	1.0	4 ± 1.15	8 ± 0.73	11 ± 0.57	95
3	1.5	3 ± 1.15	4 ± 1.15	8 ± 1.15	82
4	2.0	2 ± 0.57	4 ± 0.57	6 ± 1.73	34

Figure 4. Effect of IAA on rooting of regenerated plants.

were found resistant to spectinomycin (Figure 5), corroborating that barley is naturally resistant to spectinomycin (Figure S4).

DISCUSSION

Tissue culture and regeneration of barley (*H. vulgare* L.)

Table 6. Effect of IAA on rooting of regenerated plantlets.

S/N	Conc. of IAA (mg/L)	Average No. of roots			Rooting efficiency
		4 days	8 days	12 days	
1	0.5	2 ± 0.57	3 ± 0.57	7 ±0.57	53
2	1.0	3 ± 0.57	6 ± 1.73	9 ±0.57	95
3	1.5	2 ± 0.57	5 ± 1.73	8 ± 2.3	79
4	2.0	1 ± 0.57	3 ± 1.15	6 ± 0.57	42

Table 7. Effect of spectinomycin on genetic of cv. Haider-93.

Conc. of Spectinomycin (mg/L)	Shoot length (cm)	Root length (cm)	No. of tillers	Pigmentation
0	20.5 ± 0.28	5.5 ± 0.28	1 ± 0.57	+ + + +
25	21.3 ± 0.34	6.5 ± 1.28	1 ± 0.0	+ + + +
50	22.5 ± 0.57	7.5 ± 0.28	2 ± 0.57	+ + + +
100	21.3 ± 0.17	5.5 ± 1.28	1 ± 0.57	+ + + +
200	22.1 ± 0.31	7.5 ± 0.28	2 ± 0.57	+ + + +
300	22.1 ± 0.31	6.1 ± 0.11	2 ± 0.57	+ + + +
400	12.3 ± 0.17	2.1 ± 1.11	1 ± 0.57	+ +
500	18.5 ± 0.28	7.6 ± 0.11	2 ± 0.0	+ + +
1000	20.1 ± 0.31	9.1 ± 0.05	1 ± 0.57	+ + +

Chlorophyll pigmentation (+ +) = 25-50%, (+ + +) = 50-75%, (+ + + +) = 75-100%.

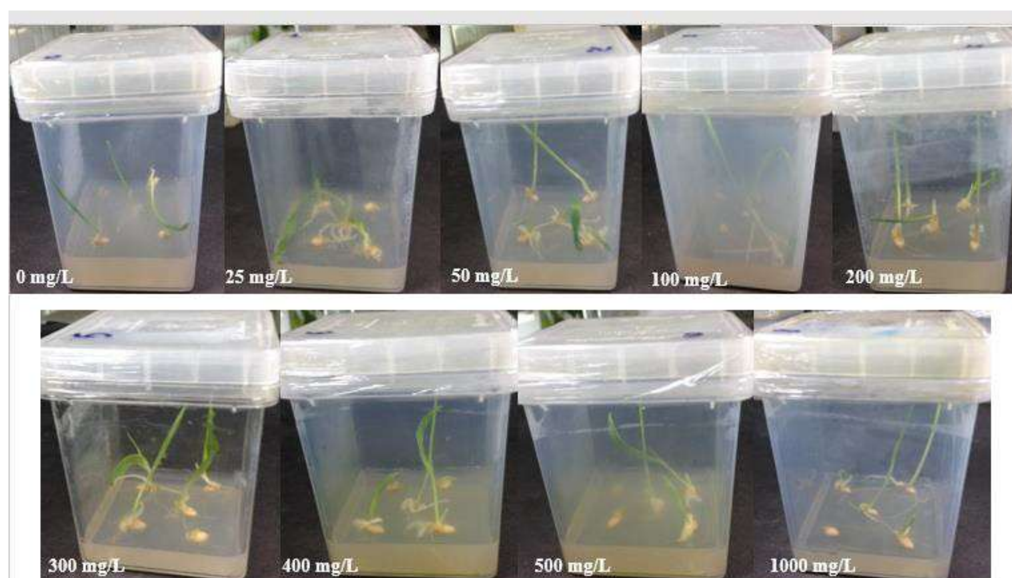

Figure 5. Effect of spectinomycin on number of tillers, shoot and root length of barley and bar = 5 cm.

are prerequisite for genetic transformation against biotic and abiotic stress resistance and biofortification. *In vitro* callus culture and regeneration of cells and tissues have broken the seasonal boundaries for production and manipulation of different crop species. Tissue culture has gained considerable attention in the recent years due to

its wide application in plant biotechnology for genetic improvement of plants. Various explants have extensively been used in establishing tissue culture protocols, such as anthers, ovaries, mature and immature embryos, etc. However, immature embryos have been reported to respond efficiently to callus induction and regeneration in comparison with mature embryos (Özgen et al., 1998; Zale et al., 2004). Using mature embryos, on the other hand, exhibits round-the-year availability which is a major advantage but low regeneration frequency is a serious drawback (Özgen et al., 1998; Yu and Wei, 2008).

Genetic improvement by both direct and indirect method is dependent on reproducible *in vitro* callus induction and regeneration system. Weights of calli induced by using immature embryos (DAP=15) as an explant on MS medium supplemented with 0.5, 1.0, 1.5, and 2.0 mg/L of 2,4-D measured as 0.414, 0.513, 0.622 and 0.837 g, respectively (Ahloowalia, 1982). Maximum callus weight was measured to be 1.798 g on MS medium containing 2.5 mg/L of 2,4-D as compared to maximum callus induction in wheat immature embryos at 2 mg/L of 2,4-D (Ozias-Akins and Vasil, 1982). However, further increase in 2,4-D concentration showed a decrease in callus weight, that is, 1.571 g on medium containing 3 mg/L of 2,4-D and so on. The present study revealed specific trend in callus induction on increasing concentration of 2,4-D. Callus weight increased with increasing 2,4-D concentration up to 2.5 mg/L. Further increase in 2,4-D resulted in decrease in average fresh weight of calli. Graphical representations of the relationship between various concentrations of 2,4-D and average fresh weight are illustrated in Table 5. More friable, embryogenic, creamy callus was observed at lower 2,4-D concentrations up to 2.5 mg/L in wheat (Ozias-Akins and Vasil, 1982). At the highest concentration, callus was more compact and yellowish in color with watery appearance (Goldstein and Kronstad, 1986).

All antibiotics have unique mode of action and exhibit different stringency against various plant species (Wilmink and Dons, 1993). Streptomycin is the most stringent antibiotic in its mode of action being tested while spectinomycin had no effect on barley (*H. vulgare* L.) regeneration because barley is a monocot. Literature suggests that streptomycin and spectinomycin irreversibly binds to the 30S ribosome and freezes the 30S initiation complex (30S-mRNA-tRNA), so that no further translation initiation occurs (Qian et al., 2012). Application of antibiotic predominantly resulted in slowdown of protein synthesis and hence induced misreading of the mRNA. Chlorosis occurs when calli are exposed to streptomycin due to severe halt in protein biosynthesis (Svab et al., 1990). Noticeably, aadA gene is responsible for conferring resistance against spectinomycin and streptomycin which poses no threat to humans or environment, thus can be potential selectable marker gene for plant transformation and also approved by European Food Safety Authority (https://www.efsa.europa.eu/en).

Immature embryos showing callus formation frequency up to 95% resulted in higher regeneration efficiency on medium supplemented with 1 mg/L kinetin is in line with previous study (Goldstein and Kronstad, 1986). The regenerated shoots were rooted on MS medium supplemented with 1 mg/L IAA (Murashige and Skoog, 1962) and acclimation was also performed. Dicotyledonous crop species upon exposure to spectinomycin show sensitivity and bleaching effect which are being successfully transformed for both nuclear and plastid transgene transformation (Day and Goldschmidt-Clermont, 2011). Spectinomycin resistance gene aadA is a valuable selection marker that is being used for the transformation of many plant species, that is, tomato, tobacco, brinjal and potato (Singh et al., 2010). In conclusion, spectinomycin is not a useful selectable marker in case of barley transformation system. The present study also revealed that barley has strong resistance against antibiotic spectinomycin up to 1000 mg/L used in culture medium.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIAL

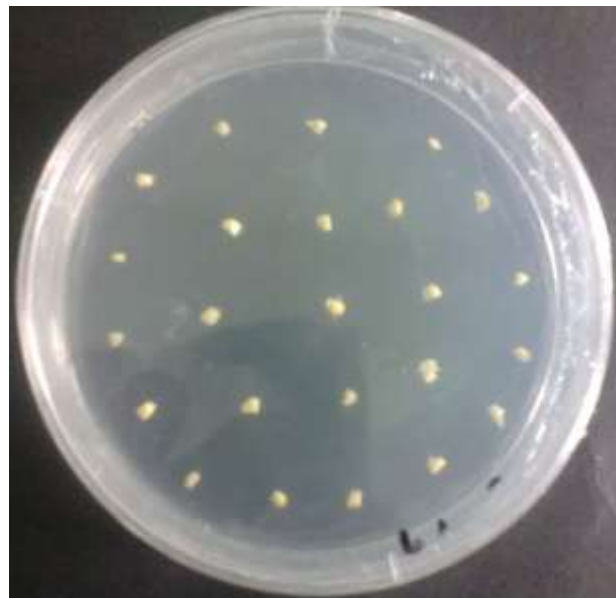

Figure S1. Callogenesis in immature embryos of barley cultured on MS + 2,4-D was started within 3-4 days.

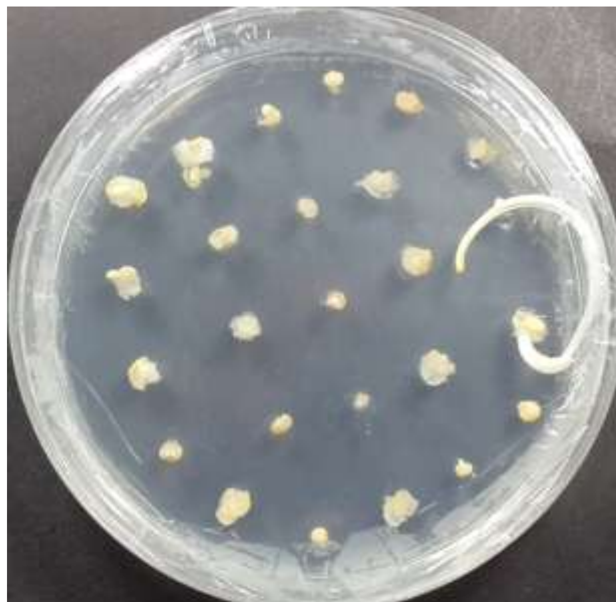

Figure S2. Shoots emerged on MS + 2,4-D removed with the help of scalpel.

Figure S3. Rooting initiation on MS + Kinetin.

Figure S4. Prospecting antibiotic resistance in barley on MS + Spectinomycin.

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Full Length Research Paper

Nutritional and physicochemical characteristics of natural fruit juice formulated from papaya (*Carica papaya*), pineapple (*Ananas comosus*) and beetroot (*Beta vulgaris*)

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Maintaining optimal nutrition and healthy population continues to be a global priority. The group of liquids and beverages plays an important role in human nutrition. Most beverages consumed are artificial and contain an array of chemical molecules that can be harmful to the health of consumers. Papaya, pineapple and beetroot are the most popular fruits and legumes in Cameroon with several therapeutical effects. This study aimed to develop natural fruit juices from the combination of papaya, pineapple, and beetroot. Four different mixed fruit juices were made and analysed for various physicochemical and nutritional properties. The results of the study showed levels of pH (4.79-5.26), carotenoid (0.01 mg/100 mL), and vitamin C (23.56-50.85 mg/100 mL) in studied juices. The moisture, carbohydrate, soluble sugar, fat and protein contents varied from 91.4-94.36%, 4.38-7.16%, 1.19-1.73%, 0.12-0.26%, and 0.51-1.24%, respectively. The minerals ranged from 0.89-1.29 mg/100 mL, 5.28-6.32 mg/100 mL and 5.26-8.13 mg/100 mL, respectively for Fe, Ca, and Mg. Amongst the four juices, J4 was shown to be the most nutritive. Juices of this study could be used as food formulation material for infants and young children to prevent iron deficiency anemia. Also, post harvest loss fruits can be reduced by converting fruit into attractive mixed juice which increase value of the product.

Key words: *Carica papaya, Ananas comosus*, *Beta vulgaris*, juices mixed, nutritional characteristics.

INTRODUCTION

Fruits and vegetables are of great importance in human nutrition (Garg et al., 2019) as their consumption maintains good health and replaces the losses in nutrients by the body (Ohwesiri et al., 2016). It is recommended for an average adult to consume about 400 g of fruits and vegetables per day to maintain good health (FAO, 2021). Fruits are good sources of essential elements which are very important for our body to make body function properly, such as water, vitamins (A, B1, B2, C, D and E), minerals (Ca, Mg, Zn, Fe, K, etc.), and

organic compounds (Begum et al., 2018). Fruits and legumes are also great sources of antioxidants which are responsible for scavenging free radicals (Ravimannan and Nisansala, 2017). In rural Cameroon, fresh fruits are consumed within short periods of seasonal availability after picked from wild trees or after harvesting. Depending on the processing technology, natural beverages may include juices or smoothies. They are produced with minimal processing and are usually consumed fresh, or soon after (Butu and Rodini, 2019).

Papaya (*Carica papaya*) is a tropical fruit with a unique flavour, aroma and pleasant sour sweet taste, a good source of carotenoid, vitamin C, and dietary fiber. It is a woody herb, growing up to 10 to 12 feet and is relatively short lived. Fruit particularly papaya, are difficult to keep for long time and are utilized either as fresh or processed into juice and specialist products due to being susceptible to bacterial and fungal contamination. Papaya juice is better for increment of acidity, thus can increase the acidity and improve the stability of juice (Patil et al., 2021). Papaya juice can enhance the flavour of juice by increasing acidity (Ewenetu and Molla, 2022).

Pineapple (*Ananas comosus*) is a widely consumed fruit grown in many tropical and subtropical regions (Debnath et al., 2021; Sun et al., 2016). Pineapple and its products are known for their pleasant aroma and flavor. The fruit is rich in certain vitamins, minerals, polyphenol antioxidants, and other phytochemicals (Ali et al., 2020). Approximately, 60% of fresh pineapple is edible, resulting in 45 to 55% of the mass of fresh fruit being discarded as waste in commercial processing operations (Da Silva et al., 2013). Phytochemical screening of the pineapple revealed the presence of saponin, glycoside, flavonoid, tanins and vitamins such as B1, B2, B3, B5, B6, B9 and C. Minerals such as calcium, magnesium, phosphorous, potassium, sodium and zinc have also been identified in the fruit. The fruit juice helps digestion (Frank and Jackson, 2014; Ikeyi et al., 2013).

Beetroot (*Beta vulgaris* L.) is a herbaceous biennial plant classified as one of the Chenopodiaceae family. The taproot found either in yellow pulp color or red where the red root utilized in salad, juice, food coloring, and as a medicine that emerged along the mediterranean coast (Kale et al., 2018; Biondo et al., 2014). Beets are considered as one of the most effective vegetables, they are a source of betalain pigment in addition to phenolic acids such as gallic, syringic, and caffeic acids and flavonoids. Beetroot is also considered as a good source of minerals such as iron, calcium, phosphorus, potassium, sodium, and zinc, in addition to vitamins like biotin, niacin, and folate. It has anti-inflammatory and antioxidant effects, which scavenge free radical from the cells promoting cancer prevention by inhibiting the tumor cells proliferation, reducing the risk of cardiovascular diseases, and expelling kidney stones (Abdo et al., 2020; Kale et al., 2018).

According to shaheel et al. (2015), the blending of fruits improve the nutritional and organoleptic qualities of the blends by synergistically contributing to human well-being when the benefits of all the fruits are combined. Juice blending has the potential to combine their individual functional characteristics to combat iron deficiency anemia. Therefore, the aim of this study was to assess the nutritional and physico-chemical characteristics of natural mixed fruit juices formulated from papaya (*C. papaya*), pineapple (*A. comosus*), and beetroot (*B. vulgaris*).

MATERIALS AND METHODS

Sample collection

The fully matured, ripe, freshly harvested papaya, pineapple and beetroot were purchased from the local market of Yaounde and then identified at the National Herbarium by comparison with the material of Betti Jean de Lagarde 243 using the specimen collection N°66220HNC for the papaya, the material of Daniel Dang 89 using the specimen collection N°18648/SRF/CAM for the pineapple, and finally with the material of Daniel Dang N°351 using the specimen collection N°25664/SRF/CAM for the beetroot.

Preparation of sample juice extraction and formulation

Preparation of papaya juice

Fresh and properly ripe papaya fruits were washed manually to remove any dust or foreign particles on their surface. After washing, the papaya were peeled and the glitches were removed. The peeled papaya were cut into small pieces and then blended in an electric blender.

Preparation of pineapple juice

For preparation of pineapple juice, fresh and ripe pineapple were used and washed with potable water. After washing, the pineapple were peeled and the cores and crown were removed. The pineapple were cut into small pieces and then blended in an electric blender.

Preparation of beetroot juice

Fresh fully ripe sound beetroot were used for extraction of pulp. After washing properly with potable water, the fruits were peeled by using knife. The beetroot were cut into small pieces and then blended in an electric blender.

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Table 1. Formulation of mixed fruit juice

Juice code	Ingredients			Total (mL)
	Papaya	Pineapple	Beetroot	
J1	33	33.5	33.5	100
J2	17	17	66	100
J3	17	66	17	100
J4	66	17	17	100

J1 = 33:33.5:33.5; J2 = 17:17:66; J3 = 17:66:17; J4 = 66:17:17 (papaya/pineapple/beetroot juice blends).
Source: Authors

Formulation and preparation of mixed fruit juice

Table 1 shows the formulation of natural mixed fruit juice with different combination of papaya juice, pineapple juice and beetroot juice. It appeared that the sample J1 contained 33% papaya juice, 33.5% pineapple juice, and 33.5% beetroot juice. The sample J2 contained 17% papaya juice, 17% pineapple juice, and 66% beetroot juice. The sample J3 contained 17% papaya juice, 66% pineapple juice and 17% beetroot juice. The sample J4 contained 66% papaya juice, 17% pineapples juice, and 17% beetroot juice. Mixed fruit juices were prepared in according to the method described by Kimouche (2008). The juice obtained was pasteurized at 90°C for 10 min. It was allowed to cool for 45 min and filled into sterile labbelled bottles and stored at -18°C. For future analysis, all four drinks were freeze-dried and stored.

Analysis of formulated mixed fruit juices

Physicochemical analysis: pH

For the measurement of the pH, the standard method of AOAC (1990) was used. The pH was determined in 10 ml of the juice dispensed into a beaker after calibration with phosphate buffer of pH 4.0 and 7.0.

Proximate analysis

The fresh mixed fruit juices were analyzed for the moisture, total nitrogen, crude lipid, crude fiber, ash, carbohydrates, and soluble sugars using the method described by the Association of Official Analytical Chemist (AOAC, 2005). Moisture content was determined gravimetrically. Approximately, 2 g of lyophilizate was weighed using a precision balance ("Sartorius"). They were dried in a "Memmert" oven at a temperature of 105°C for 24 h. Weighing was carried out regularly until a constant weight was obtained. Ash content was determined by calcination in a furnace at 550°C until it was properly ashed. The final weight of the ash was taken and ash content was calculated and analysed by dry ashing method (AOAC, 2005). The total nitrogen was determined after mineralisation of the samples according to the Kjeldahl method, followed by determination by the method of Devani et al. (1989) and then the protein content was calculated by multiplying result by 6.25. Fat content was evaluated by Soxhlet extraction according to the method described by AOAC (2005), using hexane as the extractor. Soluble sugars were extracted and determined by the 3,5-dinitrosalycilic acid (DNS) described by Fischer and Stein (1961). The total fiber content was determined gravimetrically. Carbohydrate content was determined mathematically by subtracting from 100, the percentage moisture, ash, protein, fat, and crude fiber.

% carbohydrate = 100 – (% moisture + % Protein + % fat + % ash + crude fiber).

The energy value (E) per 100 g of juice was obtained using Atwater conversion (Merill and Watt, 1955) factors as follows:

E (Kcal) = Proteins (%) × 4 + carbohydrate (%) × 4 + lipids (%) × 9 (Menezes et al., 2004).

Mineral analysis

Mineral content (Ca, Mg, Fe) of mixed fruit juices was done using atomic absorption spectrophotometric method described by Benton and Vernon (1990). In the Teflon capsules, 0.5 g of samples and 8 mL of concentrated nitric acid (97%) were introduced. After sealing, these capsules were placed in an oven at 105°C for 1 h and then cooled. After cooling, these capsules were inserted into mineralizing bombs, which were then well sealed and placed on a hot plate (130°C) for 2 h. 12 h after cooling, the mineralizations were collected in 50 mL volumetric flasks and the volumes were made up to the mark with double distilled water. The apparatus measured by directly drawing in the minerals from the stock solution. The contents were expressed in mg/100 g dry matter. After washing the capsules with distilled water, they were soaked for 12 h in a 10% nitric acid solution. They were then rinsed three times with demineralised water and dried in an oven at 60°C until completely dry.

Determination of antinutritional factors in juices

The phytate content was determined by titration with iron III solution after acid digestion (Olayeye et al., 2013). Tanins were performed using ferric reagent in an acidic alcoholic medium and gallic acid as standard (Ndhlala et al., 2007). The standard solution consisted of catechin (2 mg/mL) prepared in 70% (v/v) ethanol. After 20 min in the dark, the optical density was read at 500 nm against the blank. The tanin contents were deduced from the calibration line and expressed as mg catechin equivalent/100 mL juice. Saponin content was determined by weight difference after extraction in solvent (Koziol, 1990). Oxalate levels were determined by the method described by Aina et al. (2012). One gram of lyophilizate juices was weighed and introduced into an Erlenmeyer flask; 75 mL of H_2SO_4 (3 mol/L) was added. The mixture was magnetically stirred for 1 h followed by filtration. 25 mL of the filtrate obtained was collected and heated to 90°C and kept above 70°C at all times; the hot sample was titrated continuously with 0.05 mol /L $KMNO_4$ until a persistent pale pink color was obtained (15 s minimum). The oxalate content was then calculated by taking 1 mL of 0.05 mol/L of $KMNO_4$ as equivalent to 2.2 mg of oxalates. The results were

expressed in mg/100 mL of drink.

Determination of vitamin C and carotenoids

Vitamin C content was determined with the 2,6-dichlorophenol-indophenol (DCPIP) described by AOAC (2005) with a slight modification. Standardization of 5 mL DCP with ascorbic acid. 9.7 mg of pure vitamin C was accurately weighed out, dissolved with 50 mL of distilled water and stirred enough to dissolve all of the ascorbic acid. 5 mL of the DCP was accurately pipetted into a 50 mL Erlenmeyer flask, 1 drop of acetic acid (30%) was added to change the blue colour of DCP to a pink colour. Ascorbic acid solution was used to titrate the DCP to a colourless endpoint (or equivalence point) using burette. The volume of ascorbic acid used was recorded and the titration repeated. The quantity of vitamin C that changed the color of DCP was then calculated. Standardization process was repeated by replacing ascorbic acid solution with 5 ml of juice made up to 10 mL with distilled water. After repeating the titration 2 times, the vitamin C content was calculated from standard volume and expressed as mg ascorbic acid/100 ml of juice.

Carotenoid contents were evaluated according to the method described by Rodriguez-Amaya and Kimura (2004). Carotenoids are pigments whose color results from the presence in their structure of a multiple sequence of double bonds that absorb light between 440 and 490 nm.

Statistical analysis

The statistical analyses were carried out using R version 4.0.3 (2022-11-02) for Windows. The results of the analyses were represented as mean ± standard deviation, the tests were performed in triplicate. The significance threshold was set at 5% based on an Analysis of Variance (ANOVA) coupled with a Post Hoc test (Tukey). The principal component analysis (PCA) was used to highlight the different correlations between nutrients.

RESULTS

Physicochemical properties of juices

The results of the pH analysis of the formulated juices showed that pH ranged from 4.79 in J4 to 5.26 in J2. There was no significant difference (p>0.05) in the pH. Vitamin C content of the juice varied from 23.56 to 50.85 mg/100 mL, respectively for J2 and J4. Total carotenoid contents were 0.01 mg/100 ml for all juices.

Proximate and mineral analysis of juices

Proximate analysis and mineral content of juices are listed in Table 3. The value for moisture content ranged between 91.46 and 94.36% for J3 and J2 samples, respectively. Protein content of juices ranged from 0.51 to 1.24% with maximum value for J4 and least value for J1.

The fat content ranged from 0.12 g/100 mL in J1 to 0.26 0.14 g/100 g (J1) to 0.23 g/100 g (J3). The crude fiber content of the juice was found to be higher in the sample J2 (0.03 mg/100 g). The soluble sugar content varied from 1.09 g/100 g (J3) to 2.34 g/100 g (J2). The carbohydrate content ranged from 4.38% in J2 to 7.16% in J1. Generally, the juices were low in protein, fat, and fibre but rich in moisture. The mixed fruit juices were analysed for the presence of certain minerals such as calcium, iron, and magnesium. The iron content varied from 0.89 mg/10 0mL (J3) to 1.29 mg/100 mL (J4). Calcium content of juices ranged from 5.28 to 6.32 mg/100 mL with maximum value for J2 and least value for J3. The drinking J3 was found to have the highest amounts of magnesium (8.13 mg/100 mL).

The antinutritional factors of the juices are shown in Table 4. It appeared that oxalate, phytic acid, tanin and saponin contents were low in all the four juices. The tanin contents varied from 0.67 mg/100 g (J1) to 0.84 mg/100 g (J2). Oxalate content ranged from 1.1 mg/100 g (J2) to 2.49 mg/100 g (J3). Phytate content varied from 0.05 (J2) to 0.06 mg/100 g (J1 and J3). For the saponin contents of the four juices, they varied from 0.05 (J1) to 0.23 mg/100 g (J2). There was a significant difference between the oxalates and phytate at 95% confidence interval (p<0.05). The variables used to classify fruit juices based on their nutrient contents were performed using Principal Component Analysis (Figures 1 and 2). These figures helped to visualize the four major classes according to their nutrient formation. The variables are organized in two principal components, which express 87.75% of total variability. The axis F1 explains 51.30% of information and the second axis F2 explains 36.45% of information. It noticed that J4 is highly correlated with Fe, Mg, ash and vitamin C. On the other hand, J2 is highly correlated with the moisture and Ca analyzed in this study. J1 and J3 are in the same class, they are highly correlated with the carbohydrates, phytates, and oxalates.

DISCUSSION

Although the potential benefits fruit juices are enormous, the need to evaluate their nutritional constituents cannot be underestimated so as to provide information that may influence their choice and selection for human consumption (Owolade and Arueya, 2016). The acidic pHs are believed to be mainly due to the presence of the pineapple drink in each formulation which gives the drinks a sour taste. In the literature, pineapple has a pH of 3.5 while papaya has a pH of 4.5 and beets have pH of 5. These results are similar to those obtained by Bhavya et al. (2019) on the mixture of pineapple, beetroot, and orange drinks which varied from 3.8 to 5. The low pH is associated with the microbial stability of food because it inhibits the growth and proliferation of contaminants and thereby preserving the drink against possible microbiological alterations, for good conservation (Nwachukwu and Ezeigbo, 2013).

Total carotenoid contents were 0.01 mg/100 ml for all drinks. This result can be attributed to the composition of each drink (Bhavya et al., 2019). The carotenoid content

Table 2. Physicochemical properties of juices.

Property	J1	J2	J3	J4
pH	4.86±0.00^{a}	5.26±0.00^{a}	4.87±0.57^{a}	4.79±0.00^{a}
Carotenoids (mg/100mL)	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}
Vitamin C (mg/100mL)	36.32±0.00^{c}	23.56±0.05^{a}	32.67±0.00^{b}	50.85±0.01^{d}

Values are means ± SD of triplicate determinations. Means within the same line with different superscripts significantly different at p<0.05. J1 = 33:33.5:33.5; J2 = 17:17:66; J3 = 17:66:17; J4 = 66:17:17 (papaya/pineapple/beetroot juice blends).
Source: Authors

Table 3. Proximate analysis and mineral content of juices

Characteristics	J1	J2	J3	J4
Moisture (%)	92.05±0.94^{ab}	94.36±0.50^{b}	91.46±1.43^{a}	92.25±0.34^{ab}
Proteins (%)	0.51±0.02^{a}	0.87±0.02^{a}	0.99±0.01^{a}	1.24±0.45^{b}
Fat (%)	0.12±0.02^{a}	0.21±0.01^{b}	0.23±0.01^{b}	0.26±0.01^{b}
Ash (%)	0.14±0.02^{a}	0.15±0.01^{a}	0.22±0.01^{b}	0.20±0.01^{b}
Fibers (%)	0.02±0.00^{a}	0.03±0.00^{b}	0.02±0.00^{a}	0.02±0.02^{ab}
Soluble sugars (%)	1.73±0.02^{c}	2.34±0.04^{d}	1.09±0.02^{a}	1.19±0.03^{b}
Carbohydrates (%)	7.16±0.01^{d}	4.38±0.01^{a}	7.08±0.01^{c}	6.03±0.01^{b}
Energy (Kcal)	31.76	22.89	34.35	31.42
Fe (mg/100 mL)	0.95±0.05^{b}	0.96±0.05^{b}	0.89±0.05^{a}	1.29±0.05^{c}
Ca (mg/100 mL)	6.03±0.06^{b}	6.32±0.00^{c}	5.28±0.00^{a}	6.03±0.05^{b}
Mg (mg/100v mL)	6.13±0.05^{b}	5.26±0.05^{a}	8.13±0.05^{d}	7.87±0.01^{c}

Values are means ± SD of triplicate determinations, Means within the same line with different superscripts significantly different at p<0.05. J1 = 33:33.5:33.5; J2 = 17:17:66; J3 = 17:66:17; J4 = 66:17:17 (papaya/pineapple/beetroot juice blends).
Source: Authors

was significantly lower than that of mango nectar (2.1 mg/100 mL) (Kumar et al., 2015). Carotenoid protect cell against free radical damage (Omoregie and Osagie, 2012).

With regard to vitamin C, a significant difference is observed at the 5% threshold between the different drinks. This high content of ascorbic acid observed in J4 could be due to the high vitamin C content of the different fruits, which were used in the formulation of these drinks in addition to the contribution of each proportion. The vitamin C contents of J1, J2, J3, and J4 are similar to those found in mandarin (32.06 mg/10 mL) and soursop (20.50 mg/100 mL) drinks (Nwozol et al., 2017). However, the vitamin C content of J4 was higher than that content of the mandarin and soursop drinks. However, the levels of vitamin C in our drinks are lower than those found in orange (125.40 mg/100 mL) and lime (87.90 mg/100 mL) nectars (Chuku and Akani, 2015). Vitamin C is an extremely important nutrient for the body where it performs various functions. It helps maintain the proper functioning of the immune system to ensure the body's defenses protect cells against oxidative stress and intervenes during iron absorption. The consumption of vitamin C has also been reported to improve the rate of transformation of cholesterol, to prevent cancers and disorders associated with a lack of collagen (Fenech et al., 2019). Water is the major constituent of beverages and plays an important role in the expression of their organoleptic qualities (texture, flavor, behavior of aromas) (Ihadadene and Mahfouf, 2017). There is a significant difference at the 5% threshold between the drinks J2 and J3 shown in Table 2. This difference could be due to the proportion of fruits in each drink formulation. These values are in agreement with the FDA standard (2016). According to the latter, the water content of drinks must be greater than 80%. Moreover, our results are similar to those obtained by Tiencheu et al. (2021) on fruit drinks which varied from 79.31 to 96.84 mg/100 g. Beverages are good sources of hydration for the body.

Protein contents of our drinks ranged from 0.51 to 1.64 g/100 g. A significant difference at the 5% threshold is observed between (J1, J2, J3) and (J4). The variations observed in these values can be associated with the difference in the types of fruit used. The protein content of our different drinks was higher than that of orange (0.74 g), pineapple (0.62 g) and papaya (0.43 g) nectars (Ogbonnal et al., 2013). The general low protein content of fruit juices has also been reported for orange and pineapple drink mixed and fresh beet drink (Ohwesiri et al., 2016; Emelike et al., 2015). Proteins and their

Table 4. Antinutritional factors analysis.

Characteristics	J1	J2	J3	J4
Tanins (mg/100 g)	0.67±0.05[a]	0.70±0.00[a]	0.70±0.07[a]	0.84±0.31[a]
Oxalates (mg/100 g)	2.12±0.33[bc]	1.10±0.22[a]	2.49±0.33[c]	1.54±0.12[ab]
Phytates (mg/100 g)	0.06±0.01[a]	0.05±0.02[a]	0.06±0.01[a]	0.05±0.01[a]
Saponins (mg/100 g)	0.05±0.00[a]	0.23±0.06[b]	0.12±0.03[ab]	0.21±0.03[ab]

Values are means ± SD of triplicate determinations. Means within the same line with different superscripts significantly different at p<0.05. J1 = 33:33.5:33.5; J2 = 17:17:66; J3 = 17:66:17; J4 = 66:17:17 (papaya/pineapple/beetroot juice blends).
Source: Authors

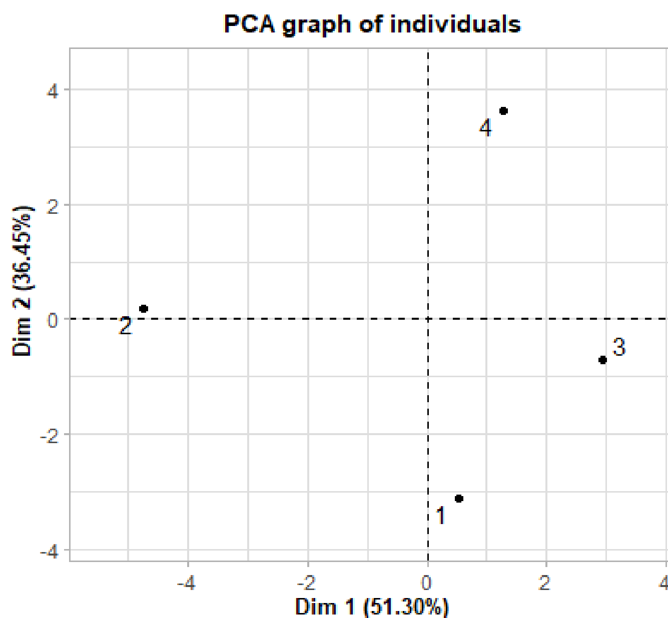

Figure 1. Distribution of the natural fruit juices formulated from papaya, pineapple and beetroot on the axis system (F1× F2).
Source: Authors

hydrolysates are important organic substances, and mineral chelating peptides have the ability to enhance the bioavailability of minerals (Guo et al., 2014). The lipid contents were from 0.12 g/100 g (J1) to 0.26 g/100 g (J4). Statistical analysis revealed a significant difference (P<0.05) between J1 and the other juices. These results are similar to those of orange (0.21 g), pineapple (0.12 g), and papaya (0.36 g) nectars (Ogbonnal et al., 2013), and lower than those of mandarin (0.86 g) and soursop (0.35 g) (Nwozol et al., 2017). This could be explained by the low lipid content of fruits of the present study. The ash contents varied from 0.14 g/100 g (J1) to 0.23 g/100 g (J3). The ash contents of juices of the present study were low to the range of 0.64-1.32% for different brands of orange juice (Ndife et al., 2013). The difference could be explained by the degree of maturity of the fruit and the growing medium conditions (temperature, irrigation and soil composition). The crude fiber contents of drinks of the present study varied from 0.02 g/100 g for drinks (J1, J3 and J4) to 0.03 g/100 g for J2. There is no significant difference (P<0.05) between these drinks. The fiber content is due to the proportion of vegetable contained in each drink. These values are lower than the value of crude fiber content of papaya (0.27 g) and pineapple (0.16 g) nectars (Ogbonnal et al., 2013). Due to its fiber content, drinks made from fruits and vegetables facilitate digestion. Crude fiber accelerates the transit of food through the digestive system and promotes the regularity or evacuation of stools (Alayande et al., 2012). Total carbohydrate contents ranged from 4.38 to 7.16 g/100 g. Carbohydrate contents (Table 2) is lower than a reported range of 8.16 to 16.19% (Ohwesiri et al., 2016) for orange and pineapple drink mixes and 7.3% (Emelike et al., 2015) for fresh beet drink. They are similar to 5.50 to

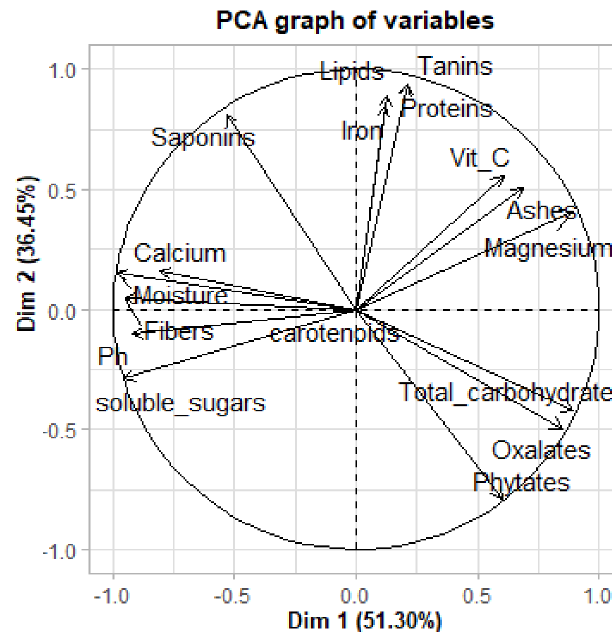

Figure 2. Correlation circle of the variables of the natural fruit juices formulated from papaya, pineapple and beetroot in the principal component analysis axis.
Source: Authors

11.80% obtained for different brands of orange drink samples (Ndifie et al., 2013). The variations observed in these values can be associated with the difference in the types and proportions of fruits used. The soluble sugar contents varied from 1.09 g/100 g (J3) to 2.34 g/100 g (J2). These values may be due to the proportion of fresh fruits contained in each drink. Sugars are the constituents that determine the sweet taste of a food, especially fruit.

In addition to providing great energy value, they play an essential role in the preservation of food products through to the osmotic pressure they exert on microorganisms and the lowering of the water activity of the food (Achir and Hammar, 2010).

The iron contents of the drinks varied from 0.89 mg/100 mL (J3) to 1.29 mg/100 mL (J4). These iron contents were high compared to beetroot, beet-pineapple and pineapple drinks mixed which were 0.214, 0.175, and 0.195 mg/100 mL (Owolade and Arueya, 2016). Iron plays a major role in cell-mediated immunity, in the control of hematopoiesis during infections and in respiratory exchanges. It is also a component of hemoglobin. Indeed, iron is important in the diet of both pregnant and breastfeeding women and children because it helps prevent iron deficiency anemia and associated diseases (Kumar et al., 2022). The iron content obtained in J4 is within the range of the RDA of iron (0.27 to 27 mg/day) (Danso et al., 2019). J4 could be recommended for optimal iron intake. In addition, through to its high vitamin C content, the non-heme iron it contains can be better absorbed. This would increase its bioavailability. The calcium content of the drinks varied from 5.28 mg/100 mL (J2) to 6.32 mg/100 mL (J3). The average calcium content of these juices is explained by the degree of maturation of our fruits and vegetables. These values are higher than those obtained by Owolade and Arueya (2016) in Nigeria on a beetroot, beetroot pineapple and pineapple drinks mixed which were 0.136, 0.182, and 0.253 mg/100 mL, respectively. Calcium is known as a macronutrient necessary for the development of teeth, bones and the release of hormones. The magnesium contents of the drinks varied from 5.26 mg/100 mL (J2) to 8.13 mg/100 mL (J3). The high magnesium content of J3 and J4 would be explained by the high magnesium content in the vegetable and the different proportions of those, which were used in the formulation of these different beverages. Magnesium is a mineral necessary for enzymes using adenosine triphosphate which contributes to DNA and RNA synthesis during cell proliferation. Magnesium deficiency causes convulsions and irritability (Achu et al., 2021). These values are higher than those obtained by Owolade and Arueya (2016).

The tanin contents of the different drinks show a significant difference at the 5% threshold. J1 had the lowest tannin content (0.67 mg/100 g). The results of this study are lower than those obtained by Nwozol et al. (2017) on the average composition of fruit drinks in Nigeria. These authors demonstrated that the tannin

contents varied from 3.73 to 67.06 mg/100 g. Similarly, the tannin contents of the different drinks are much lower than the safe dose, which is 150 to 200 mg/day (Gafar et al., 2012). This means that these drinks can be consumed without any effects. The oxalate contents varied from 1.1 mg/100 g (J2) to 2.49 mg/100 g (J3) with a significant difference at the 5% threshold. The oxalate content of J4 is lower (1.54 mg/100 g) than that contained in J1 and J3. These results were similar to those obtained by Nwozol et al. (2017) on fruit drinks in Nigeria, their oxalate contents varied from 0.05 to 2.48 mg/100 g. The presence of oxalates in food causes irritation in the mouth and interferes with the absorption of divalent minerals. These levels are much lower than the safe dose of oxalates, which is 200 to 500 mg/day (Gafar et al., 2012). Phytate levels varied from 0.05 mg/100 g (J2 and J4) to 0.06 mg/100 g (J1 and J3). These levels are low compared to that of soursop nectar (19.28 mg/100 mL) (Nwozol et al., 2017). Phytates (salts of phytic acid) represent a category of natural compounds that can have a significant influence on the functional and nutritive properties of foods of plant origin. The phytate levels in our drinks are much lower than the safe dose, which is between 2000 and 2600 mg/day (Danso et al*.,* 2019). The saponin contents of drinks of the present study varied from 0.05 mg/100 g (J1) to 0.23 mg/100 g (J2). Statistical analysis showed a significant difference ($P<0.05$) between these results. These values are similar to those obtained by Nwozol et al. (2017) which ranged from 0.03 to 3.19 mg/100 g. Saponins reduce the absorption of certain nutrients such as glucose and cholesterol in the intestine by intraluminal physicochemical interactions. The low saponin content observed could be explained by the fact that the various fruits and vegetables of the present study contain traces of antinutrients. The principal component analysis showed that J4 (66:17:17) would be the one that have high correlation with iron, vitamin C, and magnesium. Juices of the present study could play an important role in the management of iron deficiency anemia. Considering these correlations, J4 would be the one that facilitate the intestinal absorption of non-heme iron. In addition, a study by Kana et al. (2015) proves that iron and zinc supplementation can improve the biovailability of provitamin A from papaya.

Conclusion

Pineapple/Beetroot/Papaya mixed fruit juices were successfully produced and analysed for physical and chemical and properties. These juices studied here had high nutritional properties especially in terms of minerals (iron, Mg^{2+} and Ca^{2+}) moisture vitamin C and fibers. Amongst the four best formulated, juice J4 (17% pineapple, 17% beetroot, and 66% papaya) has the best content in iron, vitamin C ashes and Mg^{2+}. Combining natural fruit juices could be a good alternative to the artificial products that abound in our markets and have a negative impact on the health of the population. Our drinks could also prevent the occurrence of pathologies related to nutritional deficiencies such as iron deficiency anaemia, especially in children. These drinks are also a good alternative for processing of our products while preventing post-harvest losses.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phytochemical composition, GC-MS analysis and toxicological profiling of *Gouania longipetala* leaf extract in rats

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This study investigated the phytochemical composition and toxicity potentials of *Gouania longipetala* leaf extract in rats. Crude extract was prepared from freshly collected leaves of the plant and was subjected to phytochemical analyses including gas chromatography-mass spectrometry (GC-MS) and acute toxicity. 15 rats were used for the sub-acute toxicity evaluation, 5 rats in each group of 3, each were treated such that, group 1 is the control, with groups 2 and 3 administered 400 and 800 mg/kg of the extract orally for 28 days. Results showed the extract contains phenols, saponins, steroids, flavonoids, terpenoids with alkaloids concentration recorded as, 34.30±0.14 mg/100 g, cardiac glycoside 3.89±0.04 mg/100 g being the least. GC-MS chromatogram of the extract showed the presence of 20 compounds with Spartein (24.92%) as the most abundant and ethyl oleate (0.79%), the least. Acute toxicity (LD_{50}) value for the extract was found to be >5000 mg/kg body weight in rats. Sub-acute administration of the extract to rats did not significantly alter values of haematological and liver and renal function parameters when compared with control ($p<0.05$) but lowered levels of cholesterol and low-density lipoprotein cholesterol. Antioxidant parameters like reduced glutathione, catalase and superoxide dismutase were also increased in the extract treated rats ($p<0.05$) while Malondialdehyde concentration was lowered significantly ($p<0.05$). Results therefore showed that *G. longipetala* leaf extract may be a very safe alternative for oral use in the management of hyperlipidaemia and oxidative stress induced diseases.

Key words: *Gouania longipetala* extract, phytochemical, antioxidant, gas chromatography-mass spectrometry, toxicity.

INTRODUCTION

At the moment, alternative medicine involving the use of medicinal plants for the management of diseases appears to have been globally accepted for reasons such as availability, affordability, effectiveness and low side

effects (Ijioma et al., 2019; Okoh et al., 2019; Okoh et al., 2021). Nigeria, and in fact the whole of Africa is host to abundant forests and other natural bodies from which these medicines can be sourced (Sofowora, 1993). It is well established that phytochemical components of plants are responsible for either their healing effects or toxicity potentials (Chukwuma and Ejikeme, 2016). On one hand, the screening of plants for the purpose of providing healing has provided cheap and effective treatment alternatives for man and has in addition identified templates for new drugs discovery, but on the other hand, has exposed the toxicity potentials of plant-based medicines (Oshilonya et al., 2016), thus making toxicity evaluation a key component of medicinal plant research.

Phytochemical agents are referred to as secondary metabolites (Chukwuma and Ejikeme, 2016). Common examples are alkaloids, flavonoids, coumarins, tannins, terpenes, terpenoids, phenols, glycosides, etc. These agents possess different pharmacological activities, which determine their applications in health. For examples phenols and flavonoids are strong antioxidant agents and have been used in the fight against diseases caused by oxidative stress (Ijioma et al., 2016). The activities of flavonoids and phenols also strengthen the body's antioxidant defense line leading to reduced risk of diseases and increased well-being (Kanu et al., 2016). The presence of Vitamins C and E in plants also favors the use of plant-based medicines as promoters of the body's antioxidant defense line. For example, it is well established that vitamin C provides protection against oxidative stress-induced cellular damage by its ability to scavenge reactive oxygen species, while vitamin E plays active role in the inhibition of lipid peroxidation processes (Traber and Stevens, 2011).

Gas chromatography and mass spectrometry (GC-MS) is considered one of the most accurate bio-analytical tools available because it can identify and quantify varying active compounds including those of plant origins such as phytochemical compounds by combining the features of gas chromatography and mass spectrometry within a sample component matrix (Olivia et al., 2021). The technique has the advantage of being able to separate complex mixtures, quantify analytes, identify unknown peaks and determine trace levels of contamination (Olivia et al., 2021). Numerous medicinal plants have been screened for phytochemical composition, pharmacological activities and toxicity potentials. In this study, focus is on the plant, *Gouania longipetala*. *Gouania* is a genus of flowering plants, which belongs to the family Rhamnaceae comprising about 50 to 70 species of which *G. longipetala* is one.

These plants which are mainly shrubs are predominantly found in tropical and subtropical regions of Africa, America, and Southern Asia (Sven et al., 2011). The species *G. longipetala* is characterized by watch-spring tendrils, spike-like thyrsus, a more or less lobed disc, inferior ovary and longitudinally 3-wined septicidal fruits (Buerki et al., 2011). The stems and/or leaves of the plant are employed as alternative medicine across Africa for the management of a myriad of human ailments including but not limited to swelling, pain, edema venomous stings, gout, heart diseases, diabetes mellitus, and malaria (Njamen et al., 2013). Other areas of application of particularly the leaves are in the treatment of constipation, venereal diseases, abdominal pain and stomach upsets, pain, bacterial infections, and inflammations (Ekuadzi et al., 2012, 2014). In Orba village, South Eastern Nigeria (where the plant is referred to as "Asha"), the leaves are used specifically for the treatment of diabetes mellitus (Focho et al., 2009).

Results of scientific studies so far suggest that the plant demonstrated significant antibacterial, antioxidant, anti-inflammatory, anti-diabetic, anti-hyperlipidemic and estrogenic effects (Ezeja et al., 2015). Despite these findings, information on phytochemical composition of *G. longipetala* and its toxicity potentials remain scanty and virtually unavailable, and this is the main stay of our study here.

MATERIALS AND METHODS

Collection of plant materials and authentication

Fresh leaves of *G. longipetala* were collected from a farm settlement in Nsukka town, Nsukka Local Government Area of Enugu State, Nigeria and were authenticated at the Department of Forestry, College of Natural Resources and Environmental Management, Michael Okpara University of Agriculture, Umudike in the month of February, 2021. Dried sample of the material was assigned voucher number MOUAU/ZEB/HERB/21/006 and preserved in the herbarium of the Department of Zoology and Environmental Biology, Michael Okpara University of Agriculture, Umudike.

Preparation of extract

The Soxhlet extraction technique used by Orieke et al. (2019) was adopted with little modifications. Freshly collected leaves of the plant were dried under shade for 14 days and were thereafter pulverized to coarse powder in a manual blender. 80 g of the powdered sample was introduced into the extraction chamber of the Soxhlet extractor for extraction using ethanol as solvent. Temperature was maintained at 65°C through-out the extraction period of 48 h. At the end of the period, the collected extract in ethanol was dried in a hot air oven at 40°C to obtain a brown pasty extract which weighed 8.89 g and represented 11.11% extract yield.

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Qualitative and quantitative phytochemical analysis of *G. longipetala* leaf extract

Qualitative and quantitative phytochemical analyses of the extract were carried in accordance with the methods outlined by Ezeonu and Ejikeme (2016). The methods were used to test the presence and amounts of alkaloids, phenols, cardiac glycosides, saponins, steroids, flavonoids, terpenoids, and tannins.

Phytochemical analysis of the extract by GC-MS

The GC-MS analysis of the leaf extract was performed using BUCK M910 BUCK M910 Gas chromatography furnished with HP-5MS section (30 m long × 250 µm in width × 0.25 µm in thickness of film). Spectroscopic identification by GC–MS included an electron ionization framework, which used high energy electrons (70 eV). Unadulterated helium gas (99.995%) was utilized as the transporter gas with stream pace of 1 mL/min. The underlying temperature was set at 50 to 150°C with an expanding pace of 3°C min^{-1} and a holding season of around 10 min. At long last, the temperature was expanded to 300°C at 10°C min^{-1}. One microliter of the pre-arranged 1% of the concentrates diluted with particular solvents was infused in a splitless mode. Relative amount of the compounds present in every one of the concentrates was communicated as rate dependent on the top region created in the chromatogram.

Extraction of phytochemicals

In order to extract and separate the phytochemical components of the extract, 1 g of the sample was gauged and moved into a test tube and 15 ml of ethanol was added. The sample inside the test tube was incubated in a water shower at 60°C for 60 min. The reacting sample inside the test tube was moved to a separator pipe, following the progressive washing of the tube's sample effectively with 20 mL of ethanol, 10 mL of cold water, and 10 mL of heated water and 3 mL of hexane, which was totally moved to the channel. The combined resultant extract concentrates were then subjected to the 3 times treatment with 10 mL of 10% v/v ethanol watery arrangement. The arrangement was dried with anhydrous sodium sulfate and the dissolvable was dissipated. The sample was solubilized in 1000 µL of ethyl acetate of which 200 µL was moved to a vial for analysis.

Identification of bioactive components

The distinguishing proof of the constituents of *G. longipetala* leaf extract was accomplished on the premise of comparing the retention index of the mass spectral fragmentation patterns, with those found on the data base of the National Institute Standard and Technology (NIST). In each case the obscure spectra of the mass spectrum was compared with the known component of the NIST database.

Animals

Thirty six matured Wistar rats (120-150 g) obtained from the laboratory animal production unit of the Department of Zoology and Environmental Biology, Michael Okpara University of Agriculture, Umudike were used for the different segments of the study. Twenty-one of the rats were used for acute toxicity evaluation while the remaining fifteen were used for the sub-acute toxicity study. The rats were housed in well ventilated aluminium cages under hygienic conditions and allowed to acclimatize for 14 days before commencement of experiments. Animals were fed with normal rat chore (Chikun Finisher) and water *ad libitum* but were starved for 12 h before each experiment. All experiments were carried out in accordance with International Guidelines and as stipulated by an Ethical Committee in Michael Okpara University of Agriculture, Umudike.

Acute toxicity (LD_{50}) evaluation of *G. longipetala* leaf extract

Acute toxicity value of the extract was determined in albino rats in accordance with a modified new Lorke's method (Lorke, 1983) as was used by Orieke et al. (2019). The test was carried out in two phases. In each first phase, nine randomly selected albino rats were divided into three groups (1, 2 and 3) of three animals each and were administered 10, 100 and 1000 mg/kg body weight of the extract, respectively via the oral route. The rats were thereafter observed within 6 h post administration for signs of toxicity. With zero mortality recorded across the groups at the end of 24 h, the study proceeded into the second phase which also involved the use of a new set of 9 rats also assigned to 3 groups (1, 2 and 3) of 3 rats and administered 1600, 2900 and 5000 mg/kg of the extract, respectively. The treated animals were also observed for toxicity signs and mortalities within 24 h and a further 7 days. The metrical mean of the maximum dose that produced no mortality and the minimum dose that produced 100% mortality was taken as the mean lethal dose (LD_{50}) of the extract.

Sub-acute toxicity evaluation of *G. longipetala* leaf extract in rats

Fifteen matured albino rats assigned to 3 groups of 5 rats each were weighed and put in separate cages and assigned graded treatment with the extract as follows: Group 1: 0.5 ml normal saline (control); Group 2: 400 mg/kg *G. longipetala* leaf extract; Group 3: 800 mg/kg *G. longipetala* leaf extract.

All administrations were via the oral route and lasted for a period of 28 days before the animals were sacrificed by cervical dislocation for cardiac puncture blood collection into EDTA bottles for haematological study and also plain bottles for serum biochemical tests including liver and renal function tests and antioxidant enzymes assays. Body weights of the rats were determined at the beginning and end of treatment.

Determination of haematological, biochemical and *in vivo* antioxidant parameters

Haematological values including red blood cells count (RBCC), packed cell volume (PCV), haemoglobin (Hb), white blood cells count (WBCC), platelets count (PLTC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were determined for each sample using an automated haematology analyser (BC-2300, Mindray Company, China). Liver function parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein and bilirubin, and renal function parameters including urea, creatinine, sodium, and electrolytes were all determined using commercial test kits with strict adherence to procedures outlined by the producer Randox Laboratories, UK. Oxidative stress markers including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), and Malondialdehyde (MDA) were determined in serum using commercial test kits as was also used by Kanu et al. (2016).

Statistical analysis

Results were presented as mean values ± standard deviations (mean ± SD). The replicates in each treatment were subjected to

Table 1. Qualitative phytochemical composition of *G. longipetala* leaf extract.

Parameter	Qualitative test	Quantitative test result (mg/100 g)
Saponins	++	18.10±0.05
Tannins	+	9.72±0.25
Phenolics	+++	23.19±0.12
Flavonoids	++	16.49 ±1.08
Steroids	++	13.74±0.19
Terpenoids	+	7.45±0.10
Cardiac glycosides	+	3.89±0.04
Alkaloids	+++	34.30±0.14

+: Present.

one-way analysis of variance (ANOVA) and the difference between the samples' mean was tested by Tukey *post-hoc* test using R-statistics software version 3.03. P-values ≤ 0.05 were considered as being statistically significant.

RESULTS

Results of qualitative and quantitative phytochemical analysis of *G. longipetala*

Phytochemical agents including saponins, tannins, phenolics, flavonoids, steroids, terpenoids, glycosides and alkaloids were found to be present in *G. longipetala* leaf extract following qualitative tests (Table 1). Results of quantitative estimation of the relative amounts of these agents in the extract showed that alkaloids was most in abundance (34.30±0.14 mg/100 g) and was followed by phenols (23.19±0.12 mg/100 g) while cardiac glycosides was the least (3.89±0.04 mg/100 g). Table 1 also shows the relative amounts of identified phytochemical agents in the extract.

Results of GC-MS analysis of *G. longipetala* leaf extract

The GC-MS analysis of the ethanol extract of *G. longipetala* leaf extract as shown in the chromatogram (Figure 1) revealed 20 peaks which represent 20 identified compounds. The compounds with higher availability are Spartein (24.92%), Kamferol (12.97%) and Oleic acid (10.94%) while the compound with the least availability is ethyl oleate (0.79%). A full list of all compounds identified and quantified in the extract by GC-MS is presented in Table 2.

Result of acute toxicity evaluation of *G. longipetala* leaf extract

No death was recorded in any group at all stages of the acute toxicity test, even at the highest dose of 5000 mg/kg administered. Animals instead retained their physical activities, showed no signs of toxicity and survived throughout the 24 h and a further 7 days observation period (Tables 3 and 4). LD_{50} value for the extract was therefore found to be >5000 mg/kg body weight.

Effects of *G. longipetala* leaf extract on haematological parameters in rats

For all haematological parameters including RBC, PCV, Hb, WBC, PLT, MCV, MCH and MCHC studied, no significant difference was observed between values obtained in the test group and the control (p>0.05), although slight rise was observed in groups treated with the extract (Table 5).

Effects of *G. longipetala* leaf extract on liver and renal function parameters in rats

Treatment with *G. longipetala* leaf extract did not significantly alter the values of liver and renal function parameters in treated rats when compared with control (p>0.05). Values of total protein, AST, ALT, ALP and bilirubin did not change in the test rats when compared with control (p>0.05). Same trend of results was obtained for renal function parameters including urea, creatinine and the electrolytes when compared with control (p>0.05). Results for liver and renal function parameters are presented in Tables 6 and 7, respectively.

Effects of *G. longipetala* leaf extract on lipid profile parameters in rats

Results obtained for lipid profile in the test rats were significantly different from those obtained in the control group (p<0.05). The values of total cholesterol in the groups treated with the extract were lower than the value

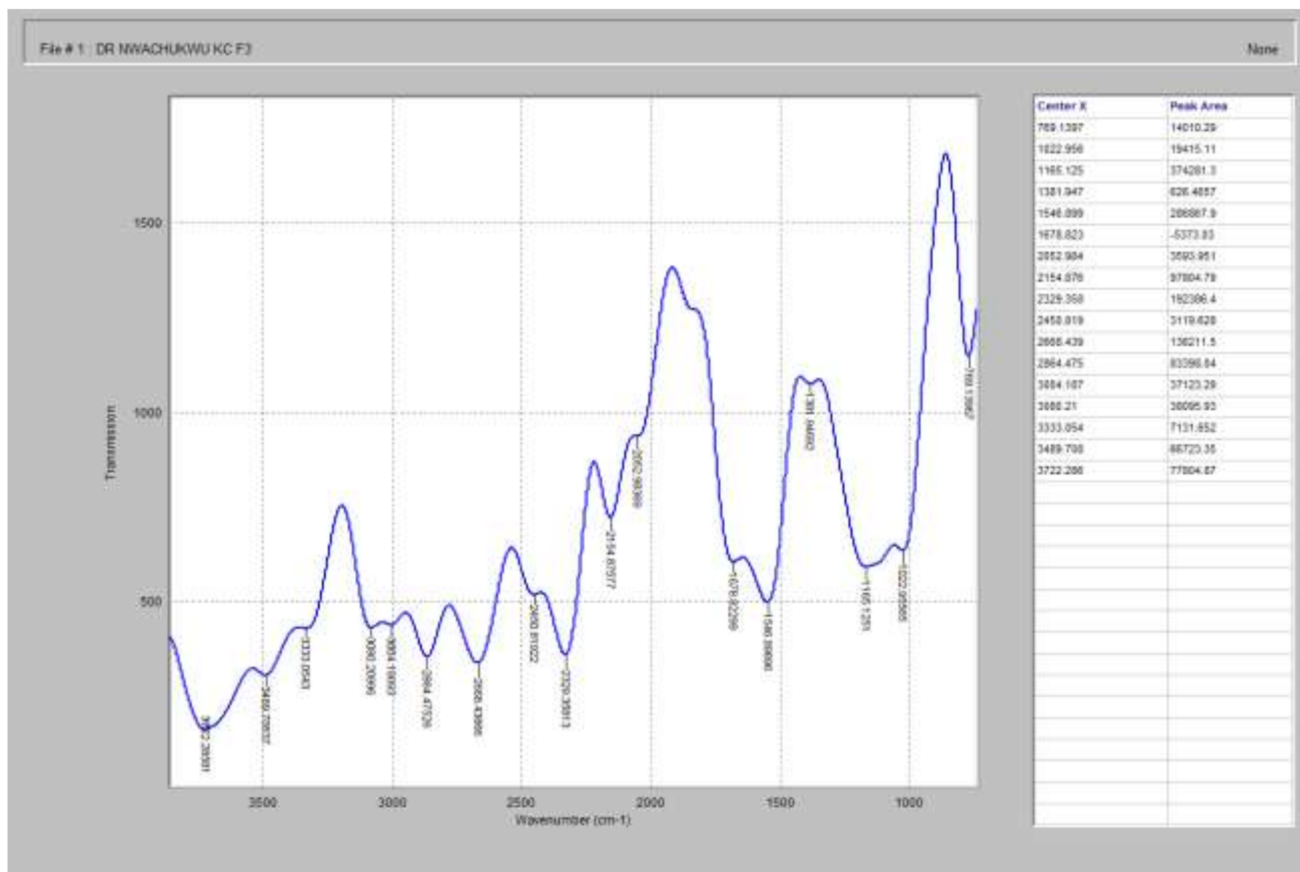

Figure 1. Chromatogram showing peaks for identified compounds in *G. longipetala* leaf extract.

Table 2. Compounds identified in *G. longipetala* leaf extract by GC-MS.

S/N	Name of compound	Concentration (%)
1	2-tetradecanol	1.07
2	Dodecanoic acid	0.98
3	1-octadecene	0.81
4	Dodecanoic acid	2.69
5	5,6-dehydrolupanine	2.11
6	Propanoic acid, 3-chloro-, methyl ester	0.66
7	Luparine	1.44
8	Sapogenine	2.14
9	Catechin	7.12
10	Flavon-3-ol	6.63
11	Anthocyanin	0.88
12	Spartein	24.92
13	Resveratrol	7.73
14	Linoelaidic acid	2.98
15	Aragyrine	4.56
16	Methyl 9,12-heptadecadienoate	2.91
17	Baptifoline	5.76
18	Oleic acid	10.94
19	Kaempferol	12.97
20	Ethyl oleate	0.79

Table 3. Phase 1 result of acute toxicity evaluation of *G.longipetala*leaf extract.

Group	Dose (mg/kg)	No. of death	Observation
1	10	0/3	Animals were active and physically stable. No signs of toxicity were observed
2	100	0/3	Animals were active and physically stable. No signs of toxicity were observed
3	1000	0/3	Animals were active and physically stable. No signs of toxicity were observed

Table 4. Phase 2 result of acute toxicity evaluation of *G.longipetala* leaf extract.

Group	Dose (mg/kg)	No. of death	Observation
1	1600	0/3	Animals were active and physically stable. No signs of toxicity were observed
2	2900	0/3	Animals were active and physically stable. No signs of toxicity were observed
3	5000	0/3	Animals were after administration, calm for about 30 minutes before regaining activity their physical activity

LD_{50}> 5000 mg/kg body weight.

Table 5. Haematological parameters in rats treated with *G. longipetala* leaf extract.

Parameter	Control	GL leaf extract (400 mg/kg)	GL leaf extract (800 mg/kg)
RBC ($\times 10^6$ mm^{-3})	7.14±0.18[a]	7.20±0.18[a]	7.29±0.29[a]
PCV (%)	44.67±0.58[a]	44.94±1.16[a]	45.33±1.53[a]
Hb (g/dl)	15.67±0.40[a]	16.10±0.36[a]	16.17±0.15[a]
WBC ($\times 10^3$ mm^{-3}))	8.71±0.39[a]	8.86±0.37[a]	8.76±0.54[a]
PLT ($\times 10^3$ mm^{-3})	194.73±6.18[a]	198.93±12.79[a]	191.80±8.45[a]
MCV (fl)	62.60±0.98[a]	62.01±0.50[a]	62.23±0.66[a]
MCH (pg)	21.95±0.04[a]	22.35±0.06[a]	22.21±0.86[a]
MCHC (g/dl)	35.07±0.61[a]	36.05±0.27[a]	35.68±1.07[a]

Values are presented as mean ± standard deviation (n = 5). The mean on the same row with different letter superscripts is significantly different (P < 0.05) from any paired value and vice versa.

obtained for the control group. Triglycerides and low-density lipoprotein cholesterol concentrations were also significantly lower than control values (P<0.05). The concentration of very low-density lipoprotein cholesterol was not altered significantly. In the evaluated lipids, only minor difference was observed (Table 8).

Effects of *G. longipetala* leaf extract on some serum antioxidant parameters in rats

Antioxidant enzymes activity levels were significantly increased in the groups treated with the extract when compared with control (p<0.05). Activities of GSH, GPx, SOD and CAT were all higher in the extract treated groups than control, but did not significantly differ at different dose levels of treatment (p>0.05). However, the concentrations of MDA in the extract treated groups are not significantly different from the control value (p>0.05). Results for these antioxidant parameters are presented in Table 9.

DISCUSSION

The significant presence of saponins, tannins, phenols, flavonoids, steroids, terpenoids, glycosides and alkaloids in *G. longipetala* leaf extract attests to the fact that the plant can be harnessed into a veritable healing substance. Phytochemical agents in plants are the reasons for their enormous medicinal potentials and have over the years been implicated in the healing of diseases (Oshilonya et al., 2016). In fact, the roles of the aforementioned phytochemical agents in the healing process have extensively been reported (Agidew, 2022; Ijioma et al., 2016; Aye et al., 2019). For example,

Table 6. Liver function parameters in rats treated with *G. longipetala* leaf extract.

Treatment	Control	GL leaf extract (400 mg/kg)	GL leaf extract (800 mg/kg)
TP (g/dl)	7.11±0.25[a]	7.30±0.09[a]	7.43±0.15[a]
ALT (U/L)	25.00±5.00[a]	27.33±3.06[a]	28.33±3.51[a]
AST (U/L)	30.00±2.00[a]	30.33±3.06[a]	34.33±2.08[a]
ALP (U/L)	84.67±4.16[a]	80.33±2.08[a]	82.33±2.89[a]
T. Bil. (mg/dl)	0.51±0.09[a]	0.55±0.03[a]	0.46±0.12[a]

Values are presented as mean ± standard deviation (n = 5). The mean on the same row with different letter superscripts is significantly different (P < 0.05) from any paired value and vice versa.

Table 7. Renal function parameters in rats treated with *G. longipetala* leaf extract.

Parameter	Control	GL leaf extract (400 mg/kg)	GL leaf extract (800 mg/kg)
Urea (mg/dl)	16.27±1.35[a]	17.27±1.06[a]	17.63±0.76[a]
Creatinine (mg/dl)	0.71±0.03[a]	0.78±0.04[a]	0.75±0.08[a]
Na^+ (mEq/L)	127.50±2.01[a]	128.77±2.17[a]	127.37±2.15[a]
K^+ (mEq/L)	4.66±0.29[a]	4.65±0.23[a]	4.97±0.12[a]
Cl^- (mEq/L)	88.13±2.27[a]	88.23±4.03[a]	88.43±3.44[a]
HCO_3^- (mmol/L)	23.17±5.32[a]	19.47±0.60[a]	19.73±0.75[a]

Values are presented as mean ± standard deviation (n = 5). The mean on the same row with different letter superscripts is significantly different (P < 0.05) from any paired value and vice versa.

Table 8. Lipid profile parameters in rats treated with *G. longipetala* leaf extract.

Parameter	Control	GL leaf extract (400 mg/kg)	GL leaf extract (800 mg/kg)
Cholesterol (mg/dl)	112.27±3.39[b]	104.80±4.77[a,b]	98.47±2.84[a]
HDL (mg/dl)	72.20±1.82[a]	73.63±1.17[a]	73.40±1.15[a]
TAG (mg/dl)	109.80±5.31[a]	108.03±3.71[a]	105.20±1.14[a]
LDL (mg/dl)	18.11±2.33[b]	9.56±4.96[a]	4.03±1.65[a]
VLDL mg/dl)	21.96±1.06[a]	21.61±0.74[a]	21.04±0.23[a]

Values are presented as mean ± standard deviation (n = 5). The mean on the same row with different letter superscripts is significantly different (P < 0.05) from any paired value and vice versa.

flavonoids and phenolics established natural antioxidants, which have been mobilized in the fight against oxidative stress diseases (Orieke et al., 2018; Foresti et al., 2005). Apart from being healing sources, some phytochemicals in plants are known to be toxic to living systems. For example, plant phytotoxin (aristolochic acid) is carcinogenic even at low doses. Some phytochemicals interfere with the absorption of food nutrients while others may be pro-oxidants and therefore may induce oxidative stress via inhibition of the antioxidant mechanisms (Yu et al., 2021), and increased formation of reactive oxygen species (ROS). This makes toxicity evaluation a paramount activity in the ongoing search for new and cheaper treatment alternative from plant sources.

The zero mortality and absence of obvious toxicity signs in this study following acute toxicity testing of *G. longipetala* leaf extract, even at 5000 mg/kg treatment dose suggests that extract from the plant may be safe for oral use in disease management. Existing guidelines for acute toxicity testing had stipulated that mortality is the expected outcome in such tests and that the observance of zero mortality within a population treated with a dose range of the substance at which mortality is expected indicates that the agent is well tolerated or that it may not be toxic (OECD, 2001). This is the basis for our conclusion on the safety of *G. longipetala* leaf extract. Similar conclusion was reached in a prototype study in which the acute toxicity effect of a plant extract was

Table 9. Serum antioxidant parameters in rats treated with *G. longipetala* leaf extract.

Parameter	Control	GL leaf extract (400 mg/kg)	GL leaf extract (800 mg/kg)
GSH (U/L)	58.33±4.51[a]	61.10±1.14[a,b]	64.80±2.26[b]
GPx (U/L)	47.87±2.29[a]	51.40±0.92[a,b]	53.73±2.12[b]
SOD (U/L)	29.67±1.42[a]	31.20±1.97[a]	34.50±0.92[b]
CAT (U/L)	19.73±2.87[a]	23.20±0.70[a]	23.90±2.87[a]
MDA (mg/dl)	0.40±0.02[a]	0.40±0.03[a]	0.41±0.03[a]

Values are presented as mean ± standard deviation (n = 5). The mean on the same row with different letter superscripts is significantly different ($P < 0.05$) from any paired value and vice versa.

established (Onoja and Anaga, 2013).

Results of the sub-acute toxicity evaluation agree with that of the acute toxicity, and further confirm the non-toxic effect of *G. longipetala* leaf extract. Values of haematological, liver function and renal function parameters in the extract treated groups are not significantly different from those of the control rats and may be an indicator of non-toxicity. Clinically, checking number of red blood cells, packed cell volume and haemoglobin concentrations have been used to assess anaemic conditions and its severity and also to monitor responses of patients to treatment, even as gross elevations in liver function parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin and renal function parameters including urea and creatinine all indicate that the liver and kidneys are diseased or are being threatened severely (Akomas et al., 2015). However, values obtained for these parameters only suggest that the extract may be safe and poses no toxicity threat to body systems. The decline observed in serum lipids (total cholesterol, triglycerides and low-density lipoprotein cholesterol) suggest that the extract may contain bioactive compounds with hypo-lipidaemic properties, which could be of value in the control of cardiovascular traumas associated with hyperlipidaemia. The results of the present study on phytochemical analysis showed the presence of saponins in *G. longipetala* leaf extract. Saponins reduce serum lipids levels by inhibition of dietary fat absorption and also by inhibiting pancreatic lipase activity (Xu et al., 2018). Improvements observed in the levels of serum antioxidant parameters like reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase may be due to the presence of flavonoids and phenolic compounds in the extract. In addition to that, natural antioxidants like vitamins C and E are also reportedly present in *G. longipetala* leaves since the extract increased the serum levels of these vitamins in treated animals (Ojobo, 2021).

Compounds identified following GC-MS analysis of *G. longipetala* have been fingered in a number of important biological activities. For example, sparteine is well known for its anti-arrhythmic activity and has been used to reduce the incidence of ventricular tachycardia and fibrillation, and also to reduce heart rate and blood pressure (Silva et al., 2014). In the pancreas, sparteine induces insulin and glucagon secretion and by that helps in the regulation of blood sugar (Fornasini et al., 2012). Its protective role against DNA damage in diabetics has also been reported (Farghaly and Hassan, 2012). Other pharmacological effects attributable to sparteine are induction of uterine contractility, and possession of diuretic, anti-inflammatory and antibacterial activities (Flores-Soto et al., 2006). Ethyl oleates are used as plasticizers and lubricants; biological additives and hydraulic fluids (34-35). Kaempferol, catechin, flavon-3-ol, anthocyanins, and other subgroups of the major secondary metabolite flavonoid identified in the extract are undoubtedly natural phenol and antioxidants which in addition to antioxidant effects are also anti-inflammatory, hypocholesterolemic, anti-cancer, nematicide, hepatoprotective, antihistaminic, antieczemic, anti-acne, 5-alpha reductase inhibitor, anti-androgenic, and anti-arthritic agent (Praveen et al., 2010). Oleic acid lowers serum concentrations of bad cholesterol but increases the good one (HDL) (Akoh and Min, 2008), thereby possesses cardio-protective activities (Hazarika et al., 2002; Gillingham, 2011).

Octadecene found in higher percentages in the hexane fraction of facultative marine fungi *Aspergillus ustus* (Oleinikova et al., 2011) and root extract of *Plumbago zeylanica* (Ajayi et al., 2011), in human and animal model studies demonstrated significant anticancer, antioxidant and antimicrobial activities (Mishra and Sree, 2007; Lee et al., 2007; Vinay-Kumar et al., 2011).

Conclusions

Findings here have shown that *G. longipetala* is safe for oral use and poses no threat to body physiology having demonstrated a high margin of safety following acute and sub-acute toxicity evaluations, practically all the parameters evaluated are within normal values. Thus, it is probable that the hypolipidaemic activity maybe

attributable to its abundant saponins composition and the other identified GC-MS compounds; albeit more research will be required to confirm this. The phytochemical components attest to the enormous healing benefits, which the plant possesses. Further studies are required to effectively harness the medicinal benefits of *G. longipetala*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Molecular characterization of probiotic *Enterococcus hirae* from fermenting *acalypha wilkesiana* (Irish Petticoat) and *Talinum triangulare* (Water Leaf) medicinal plants

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Research on lactic acid bacteria (LAB) continues to be relevant in the development of nutraceuticals. In this study, edible medicinal *Talinum triangulare* and *Acalypha wilkesiana* were collected and subjected to spontaneous fermentation for 24 h. Samples were collected at 6 h intervals for determination of pH and microbial analysis. Determination of LAB percentage frequency occurrence, probiotic properties, and safety were done. Molecular characterization of probiotic and safe LAB was carried out. During spontaneous fermentation of *T. triangulare*, pH decreased from 7.7 at 0 h to 5.8 at 24 h. Same trend was observed during *A. wilkesiana* fermentation. Microbial loads increased from 1.4 × 10^3 cfu/ml at 6 h to 4.68 × 10^7 cfu/ml at 24 h during fermentation of water leaf and from 2.56 × 10^2 cfu/ml at 6 h to 5.85 × 10^7 cfu/ml at 24 h during Irish petticoat fermentation. Forty-three LAB (30 and 13 LAB from fermenting Water leaf and Irish petticoat, respectively) were isolated in this study. They were identified as: *Enterococcus hirae* (43.3%), *Streptococcus thermophilus* (20.9%), *Enterococcus durans* (18.6%), and *Lactococcus lactis* (14.0%). However, *Enterococcus hirae* had the highest probiotic properties followed by *Streptococcus thermophilus* but the result of their molecular characterization revealed *Enterococcus hirae* and *Weisella confuse*, respectively. Considering this confusion and uncertainty about *W. confusa,* its use as a probiotic should be approached with caution. *E. hirae* is suggested to be the best probiotic potential starter for the controlled fermentation of water leaf and Irish petticoat medicinal plants. This study therefore confirms the possibility of isolating probiotic *E. hirae* from non-dairy source and suggests its incorporation into plant-based starter fermented foods.

Key words: *Enterococcus hirae*, *Weisella confusa,* probiotic, water leaf, Irish petticoat.

INTRODUCTION

Biotherapy in form of utilization of plant-based products and microorganisms in food and medicine continue to increase throughout the world due to carcinogenic related problems associated with the usage of chemical products. A medicinal plant is defined by WHO, as any plant which, in one or more of its organs, contains substances that can be used for or which is a precursor for the synthesis of useful drugs (Gopal et al., 2014).

Therapeutic properties of medicinal plants are very useful in the treatment of various diseases and the

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advantage of these plants is 100% natural with little or no side effects (Igbayilola et al., 2017). Some herbal plants like water leaf and Irish petticoat are edible hence used in the production of herbal tea because of their nutritional and therapeutic properties (Agudo, 2005; Kuete, 2017).

Talinum triangulare is a medicinal plant which belongs to the family Potulaceae and performs dual functions as food and therapeutics. It can be cooked as soup and used as medication to inhibit proliferation of cancerous cells, shrink tumors, regulate hypertension and diabetes, produce anti-inflammatory and diuretic effect, fight insomnia and enhance brain activities. According to Yilni and Naanma (2020), *T. triangulare* had numerous biological uses and functions which include suppression of oxidative damage of liver cells (Liang et al., 2011), helps to enhance liver function (Ezekwe et al., 2013), enhances cerebral, reduces oxidative stress, supports neurons of cerebrum, and enhances cerebral function (Ofusori et al., 2008), helps to treat mild laxative problem and constipation (Joshua et al., 2012), essential for managing diabetes mellitus and its component helps to slow down digestion and conversion of starch to simple sugar. *Alcalypha wikesiana* is another herbal plant with therapeutic properties. It belongs to the family Euphorbiacea. It is frequently used in traditional medicine, exclusively or as a major constituent of many herbal preparations for the management or treatment of hypertension. It is used in the treatment of malaria, dermatological and neonatal jaundice, as well as gastrointestinal disorders (Iyekowa et al., 2016).

Probiotics are “live microorganisms, which when used in adequate amounts confer a health benefit on the host” (FAO, 2011). Consumption of a large number of probiotic live microorganisms together with a food, fundamentally promotes the health of the consumers through: prevention and treatment of infectious diseases (Rolfe, 2000), curing of irritable bowel syndrome, alleviation of allergies, digestion of lactose and lowering of serum cholesterol levels (Shah, 2015) to the prevention of cancer. Probiotics are used in microbial food supplements, because they are of different human health medical been fits. As a result, probiotics have found a place in pharmaceutical plans and are provided as pharma products (Bhattacharyya, 2009). In the pharmaceutical industries, probiotics are produced as either dietary enhancement or medication. The combinations of probiotic strains give probiotic products. These probiotic products are accessible as capsule, tablets, powder, drops, bottle caps, chewing gum, etc., in the market, but mostly probiotics are available in the form of capsules (Saxelin, 2008). Most probiotics available today belong to the lactic acid bacteria (LAB) group.

Enterococci LAB are vital in food due to fermentation as probiotics in human and animals (Kamni and Ramesh, 2020). They are Gram-positive cocci, catalase-negative, non-spore formers, and occur in vegetables, plant materials, and various dairy products. Enterococci directly contribute to the distinctive flavor and taste of traditional dairy products. They also protect many fermented foods, such as sausages, cheese, and dairy products (Moreno et al., 2006). Enterococci are commonly associated with cow, goat, sheep, and buffalo milk fermentation (Quigley et al., 2013). Several species of *Enterococcus* have been commonly used as probiotics in humans and animals for the cure of gastroenteritis (Hu et al., 2015).

Enterococcus hirae is a lactic acid bacterium with reported probiotic properties such as: ability to tolerate bile, NaCl concentrations, acidic pH and phenol as well as capability to inhibit intestinal pathogens (Kamni and Ramesh, 2020).

Despite the abundance of research on the use of probiotic LAB isolated from dairy products as adjunct starter in herbal tea production, the incorporation of indigenous probiotic LAB which are thought to be better adapted to the substrate has not found the same acceptance for use in this non-dairy based functional food. This study therefore aimed to investigate the species of probiotic LAB with starter potentials for herbal tea production from fermenting water leaf and Irish petticoat.

MATERIALS AND METHODS

Sample collection

Leaf samples of water leaf and Irish petticoat were aseptically collected in triplicate from three different sources (IITA, University of Ibadan botanical garden and The Polytechnic of Ibadan botanical garden) and transported to the research laboratory of The Polytechnic, Ibadan for analysis.

Spontaneous fermentation of water leaf and Irish petticoat

Spontaneous fermentation of the leaves of water leaf and Irish petticoat were carried out according to the method of Saguibo et al. (2019) with slight modification. Twentyfive grams of the washed leaves was incorporated into 500 mL of sterile brine (containing 5% of NaCl w/v) in a glass jar with metal lid. This was incubated for 24 h at ambient conditions. Microbial analysis, pH and total titratable acidity were determined at 6 h interval for 24 h.

Isolation and purification of LAB from spontaneous fermented water leaf and Irish petticoat

Ten grams of the cut fermented leaves were De Mann Rogosa and Sharpe (MRS) agar containing 1% $CaCO_3$ using the pour plate method. The plates were incubated in an air-tight candle canister at 37°C for 48 h. Presumptive LAB colonies with clearing zones were purified by repeated streak plating using the same agar medium until pure colonies were obtained. Gram staining was carried out for preliminary identification of LAB. Further identification tests (catalase, oxidase, indole, motility and spore staining test) were carried out on the isolates.

Determination of pH

The changes in pH of fermenting samples were monitored at 6 h

intervals for 48 h using a ROHS pH meter (HANNAH instrument, Italy) (Eremosele et al., 2017).

Determination of total titratable acidity (TTA)

TTA was determined using the method described by Eremosele et al. (2017) by titrating 25 ml of supernatant from fermenting unripe plantain with 0.1 N NaOH using phenolphthalein as indicator. Triplicate determinations were carried out. TTA was determined and expressed as (Eremosele et al., 2017):

% Lactic acid = A × 0.009 × 100/v

where A=ml of 0.1 N NaOH, V=ml of sample taken for test (=25 ml).

Identification of LAB using culture dependent methods

Morphological, physiological and biochemical tests

LAB isolates were identified according to their morphological, physiological, and biochemical characteristics in line with standard techniques and this identity was confirmed using Bergey's manual of determinative bacteriology (Mulaw et al., 2019).

Probiotic characterization of LAB: Antibacterial activity of the isolates, resistance to some conventional antibiotics, tolerance to gastric acidity and resistance to bile salts were conducted according to the method described by Iyer et al. (2010).

Antibacterial activity

Antibacterial activity of the isolates was determined against *Staphylococcus aureus, Escherichia coli, Shigella* species and *Bacillus cereus.* LAB isolates were incubated for 48 h at 37°C. After incubation, cells were removed by centrifugation and pH of supernatants was set at 6.5 and it was filtered through 0.22 µm filter to obtain cell free supernatants (CFS). This CFS was used as antimicrobial agent using agar well diffusion method. Antimicrobial activity was evaluated by measuring zone of inhibition against the test organism.

Resistance to some conventional antibiotics

This was carried out according to the method of Shaikh and Gaurav (2013). The susceptibility to ten antibiotics of the LAB was investigated by the disc diffusion method on Mueller Hinton agar. After incubation at 37°C for 24 h, the diameters of inhibition zones were measured.

Tolerance to gastric acidity and resistance to bile salts

Each sample was used in triplicate to investigate the ability of the LAB to withstand gastric acidity. The bacterial pellet of young culture obtained after centrifugation (13000 rpm for 4 min) was suspended in 10 ml of MRS broth at three different pHs (2, 2.5 and 6.5). The OD from each culture obtained at 660 nm was recorded respectively at the start of the experimentation (T_{0h}) and 2 h after incubation at 37°C (T_{2h}). The number of viable cells was determined and survival rate was calculated using the following formula:

$$\text{Survival rate (\%)} = [\log UFC\ (T_{2h}) / \log UFC\ (T_{0h})] \times 100$$

The ability of the pure lactic bacteria to resist bile salt was determined. This methodology was similar to the test of tolerance to acidity described earlier except that MRS broth was supplemented with 0.3% bile salts.

Safety assessment of probiotic LAB

Safety tests such as hemolysis, gelatinase and DNase were carried out according to the method described by Nabil et al. (2004).

DNase test

This was carried out according to the method of Nabil et al. (2004). Isolates without halos-formation or clear zone formation around the colonies were negative and were selected for further studies.

β-hemolysis test

This was carried out according to the method of Nabil et al. (2004). Zones of clearing around colonies indicated β-hemolysis production. Isolates without clearance around were selected for further studies.

Molecular characterization of probiotic safe LAB

Genomic DNA extraction, PCR amplification of 16S rDNA, DNA electrophoresis, sequencing of the PCR products and phylogenetic analysis were carried out at Genoscientific Laboratory, Samonda, Ibadan with voucher number DS10031147.

Genomic DNA extraction

Genomic DNA was extracted from pure cultures of probiotic LAB. Separately, 1 ml of each pure liquid culture was centrifuged for 3 min at 10000 rpm. Supernatant was removed and the cells were suspended in 300 µl buffer (10 mM Tris-HCl, pH 8.0; 50 mM glucose, and 10 mM EDTA). To the suspension, 3 µl lysozyme (10 mg/ml) was added, and cells lysed at 37°C for 60 min under occasional stirring of the tube content by inversion. Lysing buffer (20 mM Tris-HCl, pH 8.0; 75 mM NaCl; 1% SDS; 10 mM EDTA) of 300 µl and 3 µl RNase (10 mg/ml) were added to the mixture. Mixture was incubated at 37°C for 30 min and then cooled on ice for 1 min. Ammonium acetate (7.5 M; 100 µl was added to the mixture, mixed on a vortex for 20 s and centrifuged at 13000 rpm for 5 min). Supernatant was transferred into clean 1.5 ml tubes, and 300 µl isopropanol was added. The mixture was mixed by inversion for 1 min and stored at -20°C for 30 min. Mixture was centrifuged at 13000 rpm for 5 min. The supernatant obtained was decanted, and the tubes were placed overturned on a clean filter. Four hundred microliters of 70% ethanol were added and mixed several times by overturning to wash the DNA sediment. Finally, the sediment was dried at 37°C for 15 min till ethanol drops disappeared completely. Dried sediment was dissolved in 30 µl TE buffer.

PCR amplification of 16S rDNA

For the amplification of the 16S rDNA gene, the specific primers AMP_F 5′- GAG AGT TTG ATY CTG GCT CAG -3′ and AMP_R 5′- AAG GAG GTG ATC CAR CCG CA -3′ were used. PCR reaction mixture was prepared by mixing 25 *µl* of the Taq 2x Mastermix (buffer, polymerase and dNTPs), forward primer 1 µl, reverse primer 1 µl and ultrapure water (22 µl) mixture (49 µl) was added to a sterile PCR tube, and 1 µl of the gDNA was used as a template;

Table 1. pH and total titratable acidity (TTA) of fermenting *Talinum triangulare* and *Acalypha wilkesiana*

Time (h)	Fermenting *T. triangulare*		Fermenting *A. wilkesiana*	
	pH	TTA	pH	TTA
0	7.4	0.078	7.2	0.079
6	6.1	0.084	6.8	0.088
12	6.0	0.144	6.5	0.094
18	5.5	0.151	6.2	0.116
24	5.2	0.224	5.8	0.124

Table 2. Total viable count of LAB isolated during spontaneous fermentation of *T. triangulare* and *A. wilkesiana*.

Time (h)	Total viable count of LAB (cfu/ml)	
	Fermenting *T. triangulare*	Fermenting *A. wilkesiana*
0	-	-
6	1.40×10^3	2.56×10^3
12	2.60×10^5	4.0×10^4
18	3.20×10^6	4.52×10^6
24	4.68×10^7	5.85×10^7

the amplification reaction was carried out in a thermal cycler (Bio-Rad Mycycler).

DNA electrophoresis

The PCR products was separated in a 1% agarose gel and stained with ethidium bromide followed by examination on a UV illuminator.

Sequencing of the PCR Products

A 16S rRNA PCR amplification and sequencing were performed by Eurofins, Novogene (Hong Kong). V4 hypervariable region of the16S rRNA was amplified using specific primers 515F and 806R. All the PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Libraries generated with TruSeq DNA PCR-Free Sample Preparation Kit were sequenced using paired-end Illumina sequencing (2 × 250 bp) on the HiSeq2500 platform (Illu-mina, USA).

Phylogenetic analysis

Forward and reverse sequences were assembled and edited using BioEdit Sequence Alignment Editor Version 5.0.9. Sequence similarity was estimated by searching the homology in the Genbank DNA database using BLAST. Finally, the isolates were identified based upon the sequence. Evolutionary history was inferred using the neighbor joining method. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. Tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances was computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The position containing gap or missing data was eliminated. Evolutionary analysis was conducted in MEGA7.

RESULTS

Table 1 shows the result of pH and total titratable acidity of the extracts of fermenting water leaf and Irish petticoat. The pH decreased from 7.4 at 0 h to 5.2 at 24 h while total titratable acidity increased from 0.078 to 0.224 at 48 h during fermentation of water leaf. Similar trend was observed during fermentation of Irish petticoat.

Table 2 shows the total viable count of LAB during fermentation of water leaf and Irish petticoat. The viable count increased in both fermentations from 10^3 to 10^7.

Morphological and biochemical characteristics of LAB isolated from water leaf and Irish petticoat are shown in Table 3. Fortythree LAB were isolated. They were were identified as: *Lactococcus lactis, lactis, E. hirae, Enterococcus durans* and *Streptococcus thermophilus*.

Table 4 shows percentage frequency of occurrence of LAB isolated from fermenting water leaf and Irish petticoat. *E. hirae* had the highest frequency of occurrence (43.3%) while *L. lactis* had the least (18.6%).

The antagonistic activity of LAB isolated from fermenting water leaf and Irish petticoat is presented shown in Table 5. *E. hirae* had the highest antimicrobial activity against all the tested pathogens followed by *S. thermophilus* while the safety and probiotic properties of LAB in this study are shown in Tables 6 and 7. Both *E. hirae* and *S. thermophilus* were sensitive to all the

Table 3. Morphological and biochemical characteristics of LAB isolated from *T. triangulare* and *A. wilkesiana*.

Isolate code	Morphology	Gram reaction	Catalase lase	Oxidase	Indole	motility	Endospore staining	Glucose	Fructose	Galactose	Maltose	Lactose	Mannitol	Sorbitols	Sucrose	Probable organisms
1A	Small, cream, shine, convex, circular and smooth	+ cocci	-	-	-	-	-	+	+	+	+	+	-	+	+	*Lactococcus lactis*
12A	Small, cream, shine, round, convex and smooth	+ cocci	-	-	-	-	-	+	-	-	+	+	-	+	+	*Streptococcus thermophilus*
18A	Small, cream, circular, smooth and convex	+ cocci	-	-	-	-	-	+	+	+	+	+	-	-	+	*Enterococcus durans*
43A	Medium, circular, smooth, white and convex	+ Cocci	-	-	-	-	-	+	+	+	+	+	+	+	+	*Enterococcus hirae*

antibiotics while *L. lactis* and *E. durans* were resistance to all. Also, *E. hirae* and *S. thermophilus* had the highest tolerance of bile salt (3%) while *L. lactis* and *E. durans* had the least tolerance (2%). Hemolysis and gelatinase tests as presented in Table 6 were used to assess the safety of the LAB isolates. *E. hirae* and *S. thermophilus* were negative for both tests while *L. lactis* and *E. durans* were positive to hemolysis and gelatinase tests, respectively.

Summary of molecular characterization of safe probiotic LAB and their corresponding phylogenetic tree is shown in Tables 8 and Figure 1, respectively. *E. hirae* and *S. thermophilus* were observed to be the safe probiotic LAB in this study. While the isolate 43A1 was confirmed as *E. hirae* by molecular methods, isolate 12A1 which was tentatively identified as *S. thermophilus* using conventional methods, was confirmed as *Weissella confusa* using molecular techniques.

DISCUSSION

The decrease in pH and its corresponding increase in total titratable acidity observed in this study confirm that the fermentation of *T. triangulare* and *A. wilkesiana* were carried out by acid producing isolates of which LAB is a major example among bacteria. This was in line with the findings of Eromosele et al. (2017). The increase in LAB count observed, further gives credence to the claim that the fermentation was based on LAB (Omemu et al., 2007; Onilude et al., 2008). The increase in microbial population can be attributed to their ability to utilize the available nutrient in the fermentation medium, favorable environmental condition, and absence of growth inhibitors (Augustine et al., 2019). The conventional identification of isolates which identified them as *L. lactis, Enterococcus hirae, E. durans* and *S. thermophiles* was in conformity with the reports of Adeleke and Olaniyi (2018) which stated that LAB are generally ubiquitous. They are found everywhere including air, soil and on most food substrates at low pH. The dominance of *E. hirae* in this work is supported by the reports of Adeleke et al. (2017), Ojokoh et al. (2016) and Achi and Akubor (2000). This dominance depicts the ability of *E. hirae* to produce more inhibitory substances such as acid than other fermenting organisms which tend to favor its growth and inhibit other organisms.

The high probiotic properties of *E. hirae* followed by that of *S. thermophilus* was depicted from their respective high antagonistic activity, pH, bile tolerance and lowest resistance to antibiotic as well as their safety for consumption; hence, the reason for molecular characterization of these two LAB in order to confirm species with potential starters for the fermentation of *T. triangulare* and *A. wilkesiana.*

Surprisingly, the 16s RNA molecular characterization results yielded *E. hirae* MH259885. 1 and *W. confuse* DQ321751.1*. W. confusa* and other members of the genus have been known to have similar characteristics with and difficult to differentiate from other heterofermentative *lactobacilli* based simply on morphological characteristics (Fusco et al., 2011). Many of its morphological and biochemical characteristics are similar to those possessed by the homofermentative *Streptococcus* shown in Table 3. Many commercial systems that are based on conventional techniques are not enough

Table 4. Percentage frequency of occurrence of LAB isolated from fermenting *T. triangulare* and *A. wilkesiana.*

Isolate	No. of LAB isolates	Percentage frequency of occurrence (%)
Enterococcus hirae	20	43.3
Lactococcus lactis	6	14.0
Enterococcus durans	8	18.6
Streptococcus thermophilus	9	20.9

Table 5. Antagonistic activity of LAB isolated from fermenting *T. triangulare* and A. *wilkesiana*.

LAB isolates	Susceptibility of pathogens (mm)			
	Staphylococcus aureus	***Escherichia coli***	***Shigella* spp.**	***Bacillus cereus***
Enterococcus hirae	12	10	11	9
Lactococcus lactis	6	5	6	5
Enterococcus durans	4	5	4	3
Streptococcus thermophilus	10	9	8	8

Table 6. Antibiotic susceptibility of LAB Isolated from fermenting *T. triangulare* and A. *wikesiana*.

Isolate	Zones of Inhibition of microbial growth by various antibiotics (mm)									
	APX	CN	PEF	E	SXT	S	CPX	AM	Z	R
Enterococcus hirae	1	0	0	0	1	0	0	0	0	0
Streptococcus thermophilus	0	0	2	0	0	0	1	0	0	0
Lactococcus lactis	17	12	10	14	12	10	10	15	10	12
Enterococcus durans	14	12	9	10	14	12	0	12	10	0

Table 7. Safety and probiotic properties of LAB isolated from fermenting *T. triangulare* and A. *wikesiana*.

Isolate	Hemolysis	Gelatinase	pH 1	pH 2	0% bile salt	3% bile salt	5% bile salt
Enterococcus hirae	-	-	-	+	+	+	-
Lactococcus lactis	+	-	-	+	+	-	-
Enterococcus durans	-	+	-	+	+	-	-
Streptococcus thermophilus	-	-	-	+	+	+	-

Table 8. Summary of molecular characterization of safe probiotic LAB.

Sample ID	Matched Organism	% Identity	Accession number
12A1	*Weissella confusa* strain Inje LM S-338	99.02	DQ321751.1
43A1	*Enterococcus hirae* strain HA7	93.87	MH259885.1

to completely identify members of the genus *Weissella.* Its presence in this plant based fermenting broth is not entirely novel since members of the *Weisella* genus has been isolated from plant sources such as vegetables, fruits and their fermentation based products (Cho et al., 2006; Miyashita et al., 2012; Yang et al., 2014). In line with result of this study, *W. confusa* was reported to be isolated from fermented food sources. Aveni et al. (2001) and Lee et al. (2012) discussed its potential as a probiotic which was also studied and confirmed in this

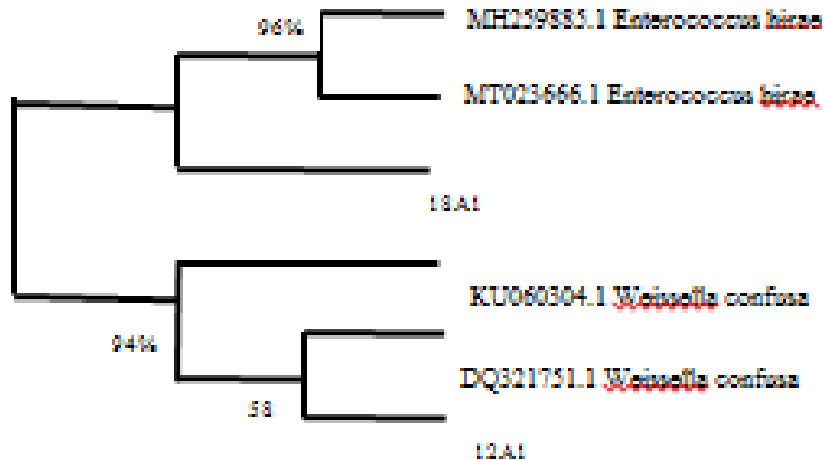

Figure 1. Phylogenetic tree of *Enterococcus hirae* MH259885.1 and *Weisella confusa* DQ321751.1.

study. The use of *W. confusa* as a probiotic should be approached with extreme caution since it has been implicated in human infections, including bacteremia.

Considering these confusions and uncertainty about *W. confusa, E. hirae* is recommended as a potential starter for the controlled fermentation of *T. triangulare* and A. *wilkesiana* medicinal plants. This is also supported by its dominance, probiotic properties and safety as observed in this work. The probiotic potential of *E. hirae* observed in this work was in line with the finding of Arokiyaraj et al. (2014), Kamni and Ramesh (2020), de Castro Santos Melo et al. (2021), and Rajput et al. (2022). Its antibiotic susceptibility contradicts the findings of Zaidi et al. (2022) who reported that antimicrobial susceptibility profiling of *E. hirae* isolates originating from beef production systems showed high resistance to tetracycline (65%) and erythromycin (57%) with 50.4% isolates harboring multi-drug resistance while it was in conformity with the work of Kamni and Ramesh (2020) who isolated *E hirae* from indigenous raw goat milk and reported that it was susceptible to amikacin, carbenicillin kanamycin, ciprofloxacin, co-trimazine, nitrofurantoin, streptomycin, and tetracycline.

Conclusion

The fermentation of *T. triangulare* and A. *wilkesiana*n was with resultant dropped pH and increased population of LAB showed that it is LAB fermentation. Out of four species of probiotics obtained in this study, only isolate 12A1 and 43A1 were safe for consumption hence the reason for their molecular characterization. Isolate 12A1 which was tentatively identified as *S. thermophilus* using conventional methods, was confirmed as *W. confusa* using molecular techniques while isolate 43A1 was confirmed as *E. hirae* by both conventional and molecular methods, considering confusion and uncertainty of *W. confusa, E. hirae* is therefore suggested to be a potential starter for the controlled fermentation of *T. triangulare* and A. *wilkesiana* medicinal plants. It can therefore be concluded from result of this study that *T. triangulare* and A. *wilkesiana* medicinal plants are sources of potential starter probiotic *E hirae* MH259885.1 which met the criteria to be considered as safe probiotic for application in food fermentation.

The role of *E. hirae* in starter produced medicinal plants is recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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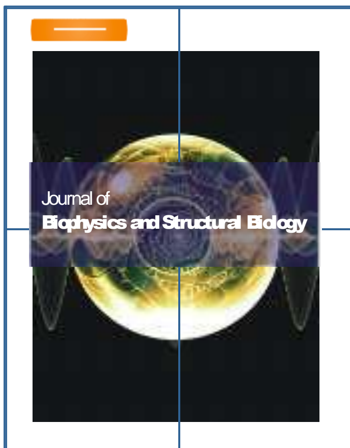

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