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Full Length Research Paper

Encapsulation and gastrointestinal endurance of *Lactobacillus reuteri* DSM 17938 strain with emulsion polymerization

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***L. reuteri* DSM 17938 strain was encapsulated using emulsion polymerization technique with alginate and fructooligosaccharide at five different concentrations between 0 and 1.5%. This study aimed to improve the gastrointestinal system (GIS) viability of *L. reuteri*. Encapsulation yield was calculated and found to be between 98.67 and 86.88%, and SEM imaging was performed for beads, and their sizes were found to range from 68.81 µm to 351.0 µm. In addition, microbial growth in GIS was indicated for 3 h at intervals of one hour. 0.75% fructooligosaccharide plus 2% alginate capsules yielded the highest viability in a simulated gastric environment. At the end of 3 h, these capsules were decreased 0.39±0.03 logarithmic cycle, but the non-encapsulated control sample was decreased 2.10±0.16 log. The control sample was decreased by 5.8 log cycle in the simulated bile environment, but capsules were decreased by 2.5-3.4 log cycle on average. The result was statistically significant and showed that the encapsulation process protected the survival of microorganisms in GIS.**

Key words: Encapsulation, gastrointestinal system, lactic acid bacteria, survival rate.

INTRODUCTION

Probiotic microorganisms are defined as living microorganisms that provide health benefit to the host when they are sufficiently taken by the body (FAO and WHO, 2006; Muthukumarasamy et al., 2006; Ünal and Erginkaya, 2010).

Benefits of probiotics to the host are as follows: adjustment of the immune system, synthesis of the components that provide anti-cholesterol characteristics, anti-carcinogenic effects, improvement of intestinal flora with the antimicrobial materials they produce (Muthukumarasamy et al., 2006; Schell and Beermann, 2014), treatment of some intestinal disorders, positive impact on systemic diseases like allergy or inflammatory diseases, and positive effects in the treatment of vaginitis (Martin et al., 2015). *Lactobacillus reuteri* is an obligate heterofermentative, gram-positive, and catalase-negative lactic acid bacterium (Ünal and Erginkaya, 2010). Being indigenous to the human intestinal system and well-

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colonized in intestines, this probiotic microorganism shows a preventive effect on the diseases originating from pathogens (Muthukumarasamy et al., 2006; Gu et al., 2015). *L. reuteri* can synthesize reuterin, known as beta-3-hidroxypropionaldehyde, from glycerol in anaerobic conditions. Reuterin is a neutral metabolite with a non-protein structure. It is resistive against various proteolytic and lipolytic enzymes and effective in a wide range of pH, and shows an antimicrobial effect against *Escherichia coli* O157:H7, yeast, mold, and protozoon (Mohammed et al., 2020). *L. reuteri* DSM 17938 is the daughter strain of *L. reuteri* ATCC 55730 strains that has transferable resistance traits for lincomycin and tetracycline (Urbańska and Szajewska, 2014). It has a positive effect on the treatment of diarrhea (Dinleyici et al., 2015) and colic complaint (Szajewska et al., 2013).

Probiotic foods include 10^8-10^9 cfu/ml alive microorganism (Shori, 2017) and a minimum of 10^6-10^7 cfu/ml microorganism is colonized in intestines by passing through digestive system during the entire shelf life of the product (Ünal and Erginkaya, 2010; Martin et al., 2015; Shori, 2017). The main problem with consuming these products is that the microorganisms cannot maintain their viability during shelf life and in the GIS. Supporting probiotic microorganisms with a physical barrier such as encapsulation will positively impact their viability (Ünal and Erginkaya, 2010; Martin et al., 2015). Many of the studies show that the probiotics that are encapsulated with biopolymer matrix improve the strength of the GIS (Krasaekoopt and Watcharapoka, 2014; Rodklongtan et al., 2014; Villena et al., 2015; García-Ceja et al., 2015; Martin et al., 2015). The most suitable encapsulation methods for probiotic microorganisms are emulsion, extrusion (Muthukumarasamy et al., 2006), and spray drying (Pankasemsuk et al., 2016; Ünal and Erginkaya, 2010). Extrusion technology has advantages like easier application and more uniform capsules than emulsion (Martin et al., 2013; Muthukumarasamy et al., 2006), but it has a disadvantage in that, the larger-sized capsules cause a decrease in sensory quality. On the other hand, spray drying process conditions cannot be controlled totally (Gökmen et al., 2012).

In addition, the properties of the wall material to be selected are essential for the efficiency and reliability of the encapsulation (Naveena and Nagaraju, 2020).

De Prisco et al. (2015) encapsulated *L. reuteri* DSM 17938 strain by alginate and alginate-chitosan matrix and exposed to different stress conditions. Non-capsulated controls were decreased by 2.09 logarithmic phase in 3 h, which simulated gastric environment, but alginate capsules were decreased by 0.35 logarithmic phase. García-Ceja et al. (2015) capsulated *Lactobacillus acidophilus* and *L. reuteri* individually and in combination by using alginate and alginate-chitosan system. They reported that alginate-chitosan capsules had a better gastrointestinal system resistance compared to the alginate capsules, and the combined encapsulation of lactobacilli could provide more health benefits than individual encapsulation. Muthukumarasamy et al. (2006) encapsulated five different *L. reuteri* strain by using alginate with starch, κ-carrageenan with locust bean gum, and xanthan with gallant gum and studied the endurance in simulated gastric conditions. It was reported that all the capsulated samples had a better endurance compared to the control, and alginate and alginate plus starch yielded the best positive results.

The presence of prebiotics such as various oligosaccharides in the environment was reported to increase the viability and resistance of probiotics in the gastrointestinal tract (Burgain et al., 2011; Rajam and Anandharamakrishnan, 2015). Prebiotics are resistant in the gastric environment and partially fragmented by intestinal enzymes in the intestine.

Fragmentation products are a substrate for the fermentation of probiotic microorganisms. So, the combined use of probiotics and prebiotics shows a synergistic effect. Inulin, fructooligosaccharide, and galacto-oligosaccharide are the compounds that are used in this manner (Rajam and Anandharamakrishnan, 2015) and show a prebiotic impact (Rajam and Anandharamakrishnan, 2015; Dias et al., 2018).

Although there is much information about *L. reuteri* DSM 17938 strain in medical literature, there is a limited number of food studies on the use of this organism. The authors aimed to use alginate in combination with FOS as wall materials for the encapsulation of *L. reuteri*. The effect of wall material combination, encapsulation efficiency, the morphology of microcapsules, and viability of cells in gastrointestinal (simulated gastric and intestinal) conditions was evaluated.

MATERIALS AND METHODS

Preparation of probiotic culture

Freeze-dried *L. reuteri* DSM 17938 (BioGaia AB, Stockholm/ Sweden) was activated twice by deManRogosaSharpe (MRS) broth (Merck, Germany) under anaerobic conditions at 37ºC. In the first activation, microorganisms were washed with sterile 0.1% peptone (Merck, Germany) water. Then, the culture was inoculated to 5 ml MRS broth medium for 24 h. The culture was transferred into MRS broth and then incubated for 18 h. After the incubation, the sample was centrifuged for 10 min at 4500 rcf and washed with peptone water two times. The culture was diluted to 10^{10} colony forming units per ml (cfu/ml) concentration with 0.1% peptone water.

Encapsulation of probiotic cells

L. reuteri DSM 17938 was entrapped by an emulsion technique following the method of Apichartsrangkoon et al. (2015). Sodium alginate (Carl Roth, Germany) and sodium alginate containing 0, 0.5, 0.75, 1, or 1.5% fructooligosaccharide (Sigma, America; Average degree of polymerization of >10) were used as covering material. Accordingly, 40 ml sterile covering material was mixed

Table 1. Size of the beads and efficiency of the encapsulation.

Covering material	Efficiency of encapsulation	Size of capsule
2% alginate	98.67±3.30[a]	178-292 µm [a]
0.5 FOS added 2% alginate	87.71±2.14[b]	68.81-232 µm [b]
0.75 FOS added 2% alginate	87.19±0.81[b]	119-248 µm [b]
1 FOS added 2% alginate	86.88±1.12 [b]	98-351 µm [b]
1.5 FOS added 2% alginate	88.83±1.25 [b]	140-208 µm [b]

Average Value±Standard Deviation, averages showed in the same column with a different letter are different from each other (p≤0.05).

with 10 ml of the culture and 200 ml of sterile sunflower oil (Torku, Turkey) containing 0.2% (v/v) Tween 80 (Merck, Germany) and stirred for 20 min at 450 rpm. Tween 80 used as emulsifying agents provides a better homogenization by lowering the interfacial tension of the two immiscible phases and can be used to prepare smaller capsules (Pech-Canul et al., 2020). After that, 200 mL of sterile 0.1M $CaCl_2$ (Merck, Germany) was gently added into the mixed solution and stirred for 10 min at 350 rpm. Formed beads were separated by 110 micron filter paper under vacuum and dried at freeze dryer (Christ Alpha 1-2 LD) for one night.

Encapsulation efficiency

To identify the number of bacteria after the encapsulation, resolution of beads was provided by mixing 100 mg lyophilized bead and 9 ml EDTA for 20 min at 450 rpm (Tsen et al., 2007). The encapsulation efficiency (EE) was calculated using the following formula based on the ratio of the number of live cells released from the beads to the initial number of cells.

$$Survival\ rate = \frac{N_0}{N_1} \times 100 \quad (1)$$

N_0 = Viable bacteria after encapsulation (log cfu/ml); N_1 = Viable bacteria before encapsulation (log cfu/ml)

SEM Screening of capsules

After freeze-drying, measuring the size of the beads and the morphology of the microcapsules was examined using scanning electron microscopy (SEM, Zeiss EVO HD 15) at an accelerating voltage of 15.0 kV. The capsulated samples were taken from the modules of SEM screening device, and their upper surfaces were covered with gold-palladium before analysis. Representative SEM images for all beads were determined.

Simulated gastric conditions

A simulated gastric solution (SGS) was prepared according to De Prisco et al. (2015). The solution was prepared with 5 g/L NaCl (Merck, Germany) and 3 g/L pepsin enzyme (Fisher, UK), and pH was adjusted to 2.5 with concentrated HCl (Merck, Germany). The gastric solution was sterilized by filtration (0.10 µm). Aliquots of 0.1 g of encapsulated cells or 0.1 mL of free cell suspensions (10^9 cfu/ml) were mixed with 9 mL of SGS and incubated for 60, 120, and 180 min at 37ºC. The capsulated and free cells were inoculated on MRS agar (Merck, Germany). Before the inoculation, the SGS was removed and then 10 ml 4% EDTA (abcr, Germany) solution was added for the dissolution of capsules at 450 rpm for 20 min (Tsen et al., 2007).

Simulated bile conditions

Three different simulated bile solutions (SBS) were prepared and tested. The first bile solution was prepared as 0.05M KH_2PO_4 (Merck, Germany) that included 6 g/L bile salt (Sigma-Aldrich, America) at 7.5 pH (Krasaekoopt et al., 2004). The second bile solution was designed as described by De Prisco et al. (2015). 5g/L bile solution was used for this. The third bile solution was prepared, according to Rodklongtan et al. (2014), as a phosphate-buffered compound that involved 0.3% bile salt. Additionally, the bile bladder of 4-year-old Simmental cattle was used as control.

Bile solution was sterilized by filtration (0.10 µm). Aliquots of 0.1g of encapsulated cells or 0.1 mL of free cell suspensions (10^9 cfu/ml) were mixed with 9 mL of SBS. Capsulated and free cells were incubated in SBS for 3 h and inoculated on MRS agar at intervals of one hour by the cultural way. Before the inoculation, the capsules were dissolved by the same way applied in the Section Simulated Gastric Conditions. Simulated gastric and bile resistances were examined individually.

Statistical analysis

Statistical analysis of the data was carried out using IBM SPSS Statistics software (v.24). Differences between the results were identified using One-Way ANOVA method and Tukey multiple comparison tests. The statistical significance was set at (p≤0.05).

RESULTS AND DISCUSSION

Efficiency of encapsulation and size of capsules

Table 1 gives the average size of the beads and the efficiency of the encapsulation. The results showed that all the encapsulated cells were able to grow, and no significant or minor damage was recorded during the encapsulation. While the highest capsulation efficiency was found to be 2% alginate with 98.63±3.30%, the lowest efficiency was observed in alginate plus 1% FOS beads with 86.88±1.12%. The results indicate that the microencapsulation in the alginate beads was more appropriate for microencapsulation of *L. reuteri* than the alginate plus FOS capsules. Krasaekoopt et al. (2003) reported that vitality proportion of probiotic micro-organisms was between 80 and 95% in the emulsion technique.

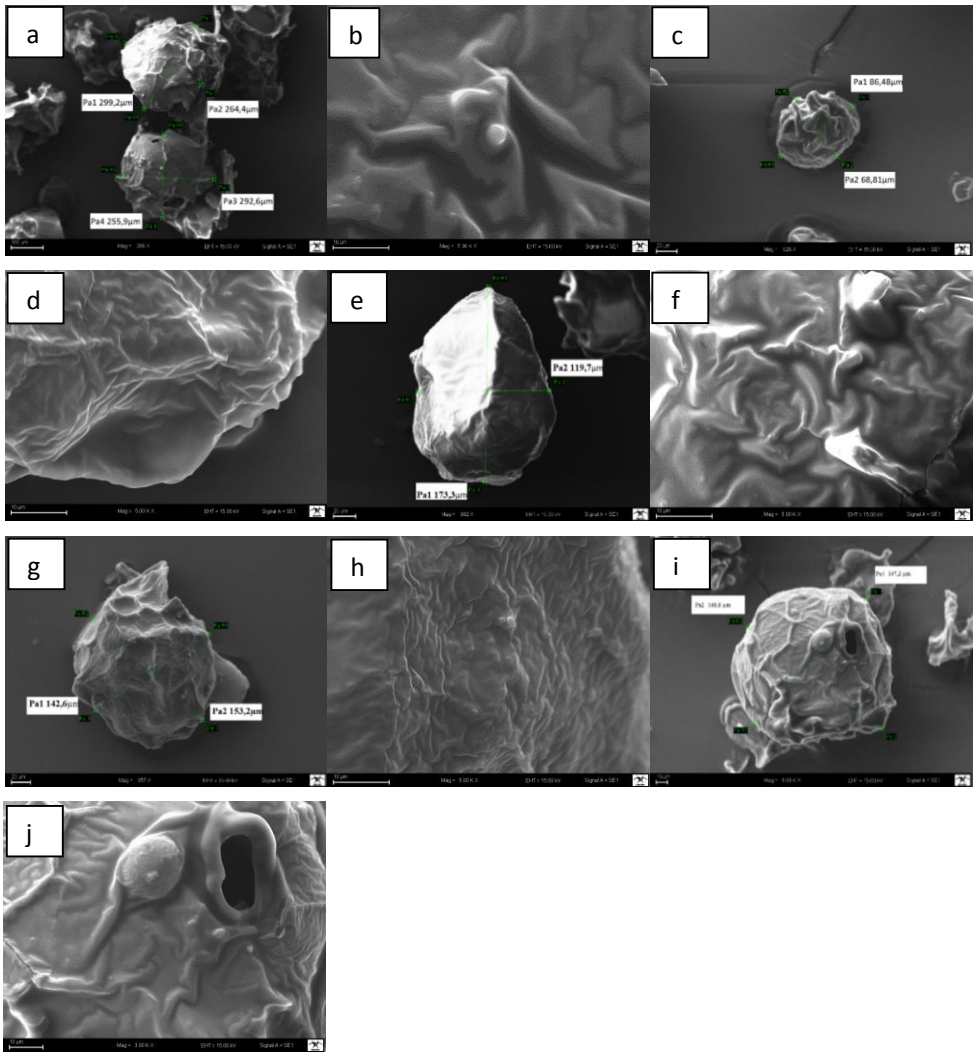

Figure 1. a. 2% Alginate Capsules b. Capsule Surface of 2% Alginate Capsules c. 0.5% FOS Added 2% Alginate Capsules d. Capsule Surface of 0.5% FOS added 2% Alginate Capsules e. 0.75% FOS Added 2% Alginate Capsules f. Capsule Surface of 0.75 FOS% Added 2% Alginate Capsules g. 1% FOS Added 2% Alginate Capsules h. Capsule Surface of 1% FOS Added 2% Alginate Capsules i. 1.5 FOS% Added 2% Alginate Capsules j. Capsule Surface of 1.5% FOS Added 2% Alginate Capsules.

Bilenler et al. (2017) capsulated *L. plantarum* with alginate plus starch wall material at different proportions and found similar capsulation efficiency. A previous study examining the encapsulation efficiency for *Lactobacillus fermentum* CECT5716 reported less survival with the same technique in alginate (Martin et al., 2013).

The size of the beads and their surface structure were determined by SEM Screening. Thus, five particles were measured from each capsule. SEM images were illustrated in Figure 1. The beads had an almost spherical shape and a disordered structure. Moreover, it was determined that the surfaces of the beads were wrinkled during the SEM screening, and when it was zoomed, the beads showed no significant diversity in terms of surface morphology.

The average size of the beads was as follows; the alginate beads, 235±30 µm; alginate plus 0.5% FOS beads, 150±52 µm; alginate plus 0.75% FOS beads, 183±41 µm; alginate plus 1% FOS beads, 173±59 µm; and alginate plus 1.5% FOS beads, 174±25 µm. Bead sizes for each capsule were illustrated in Figure 2. 2% alginate beads were found to have a statistically significantly bigger size than the alginate with FOS beads ($p<0.05$). Similar results were found in a study carried out by Krasaekoopt and Watcharapoka (2014). The sizes of capsules were reported as 1.90-1.92 mm in prebiotic supplementary alginate capsules.

Similarly, Gandomi et al. (2016) reported the sizes of alginate and alginate with inulin capsules as 1.39 mm and 1.40 mm, respectively. The sizes of capsules

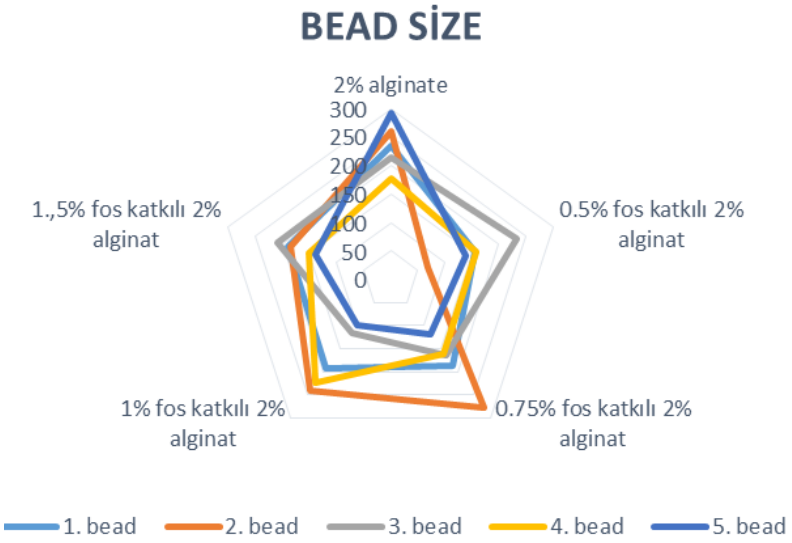

Figure 2. Beads size from each of the capsules.

Table 2. Survival rate of probiotic culture after application.

Capsule material	Survival rate of probiotic culture after 3 h SGF application (%)		
	1 h	2 h	3 h
Control	96.31±1.3 ^{a,x}	84.92±2.3 ^{a,y}	78.48±1.8 ^{a,z}
2% Alginate	96.93±1.5^{a,x}	96.43±2.2 ^{b,x}	83.89±1.0 ^{b,y}
0.5% FOS Added Alginate	96.22±2.0^{a,x}	94.16±2.5 ^{c,y}	92.26±1.1 ^{d,z}
0.75% FOS Added Alginate	97.73±1.4^{a,x}	96.12±1.4 ^{b,x}	95.92±1.6 ^{c,y}
1% FOS Added Alginate	98.19±1.1^{a,x}	94.64±1.3 ^{b,y}	92.42±0.4 ^{d,z}
1.5% FOS Added Alginate	95.90±1.2^{a,x}	94.17±1.3 ^{c,y}	92.54±1.9 ^{d,z}

Average±Standard Deviation; the first letter states the meaningfulness of difference between columns, and the second letter states it for lines. The different letter shows that meaningful difference occurs (<0.05).

reported by Prasanna and Charalampopoulos (2018) were bigger than those in the present study. They found the sizes of sodium alginate-cow milk and sodium alginate-goat milk capsules as 2.8±0.3 mm and 3.1±0.2 mm, respectively. Martin et al. (2015) determined the size of alginate capsules as 30-60 µm. The reason why the sizes of the capsules were smaller than those in similar studies may be the addition of $CaCl_2$ while crushing the emulsion.

Endurance of capsules in gastric environment

Gastric environment tolerance of the capsulated cells and the control were shown in Table 2. Low viable cell counts were determined when the alginate was used alone. FOS was used in the mixture to obtain a more strong and stable wall structure. After 3 h of treatment, 0.39±0.03 log cycle decrease was observed in 0.75% FOS added alginate capsules and 0.76±0.30 log cycle decrease in 2% alginate capsules, and the other results were between these values.

On the other hand, a 2.10±0.16 log cycle decrease was observed in the control samples. A negative correlation was found between the increase in processing time and the number of the alive microorganisms. Besides, according to the one-way ANOVA results, 0.75% FOS + 2% alginate capsule was found to be the best in terms of keeping the liveliness of microorganisms in SGS ($p<0.05$). There was no significant difference between the capsules of 2% alginate plus 0.5, 1, and 1.5 FOS ($p>0.05$). The previous studies in the literature reported results similar to ours for *L. reuteri* survival in SGS. Muthukumarasamy et al. (2006) examined the SGS resistance of 6 different *L. reuteri* strains and reported a 0.28-1.26 log cycle decrease. Similarly, Zhao et al. (2012) reported a decrease of <1 log cycle as a result of 2 h of SGS treatment on *L. reuteri* coated with alginate

capsules. Likewise our results, numerous studies reported that the capsulation of microorganisms improved the SGS endurance (Rodklongtan et al., 2014; Apichartsrangkoon et al., 2015; De Prisco et al., 2015; Villena et al., 2015; Shori, 2017).

Endurance of capsules in bile environment

The capsules and control samples in the simulated bile environment were unable to maintain their viability. No microbial viability was observed due to the lack of nutrients in the environment or using harsh bile conditions. For bile liquid, a 4-year-old Simmental cattle's gallbladder was taken without disintegrating, and bile was removed in aseptic conditions. It was determined that after the treatment of this bile liquid for 3 h, 64.05-66.58% of the capsules were alive. While the capsulated samples were decreased to 2.53-3.48 logarithmic phase, the control sample was decreased to an average of 5.81 logarithmic phases. This difference was found to be statistically significant ($p<0.05$). It was determined that encapsulation increased the SBS resistance of *L. reuteri* DSM 17938. Some studies reported a better survival for *L. reuteri* encapsulated in alginate than our study. For example, De Prisco et al. (2015) reported a reduction of 0.82 log phase for Lactobacillus reuteri DSM 17938 in 4 h. On the other hand, Gülsünoğlu (2013) observed a reduction of 2.24 log in SBS for *L. acidophilus* LA5 in 4 h.

CONCLUSION AND RECOMMENDATIONS

In recent years, there is a growing interest in functional foods, especially probiotic products. However, these microorganisms lose their vitality under adverse environmental conditions. This both restricts probiotics in products such as dry food and reduces the shelf life of products that include probiotics. Scientists work on improving the endurance of microorganisms under different adverse conditions. Encapsulation can improve the resistance of microorganisms against drying, gastrointestinal system, and heat treatments like freezing and heating.

The coated material was successfully used as a wall system to encapsulate *L. reuteri* DSM 17938 by emulsion polymerization, and an encapsulation yield higher than 86% was obtained. In the encapsulation process, 0,5% FOS added 2% alginate capsule was found to yield the least vitality, whereas the highest vitality was observed in 2% alginate capsules. Gastrointestinal resistance of this strain was increased by alginate and FOS added alginate capsules. As a result of the statistical analysis, it was found that 0.75% FOS plus 2% alginate capsule was the best coating material and 2% alginate capsule was the worst coating material in terms of GIS viability. However, all the capsule materials protected the *L. reuteri* DSM 17938 strain better than the control. When the SBS resistance was examined, it was found that the encapsulated cells were better preserved than the control. FOS added capsule material was found to have a positive effect on viability. In addition, a negative correlation was found between viability and processing time in all the samples, including the control. In conclusion, the encapsulation was carried out successfully, and the gastrointestinal system resistance of *L. reuteri* DSM 17938 strain was improved with encapsulation.

There is an increasing interest in the studies on encapsulation with prebiotics. It is thought that this microorganism can be used as a probiotic additive in functional foods. In future studies, the authors plan to use different prebiotic or carbohydrate basic capsules and examine different probiotics together to see whether they will create a synergistic effect.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interest.

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Evaluation of chemical composition of *Dacryodes edulis* (african pear) seed oil at different stages of fruit maturation

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Nutritional and industrial processes have increased the demand for oils and this in turn has led to the search for oils from different types of seeds for possible development and use. It is in this vein that the fruit pulp of *Dacryodes edulis* was extracted with n-hexane (soxhlet extraction at 65°C). The proximate composition, antinutrient and mineral content of freshly harvested *D. edulis* fruit pulp from 4 weeks after anthesis (WAA) to fruit maturation were assessed in this study. Data obtained for the proximate composition at matured stages of fruit development revealed high amount of fat (53.4 ± 1.35%; moisture (47.2 ± 1.20%); crude protein (17.0 ± 0.48%); carbohydrate (21.70 ± 0.98%); low crude fiber (1.60 ± 0.75%) and ash content (8.0 ± 0.81%) which contained higher amount of minerals such as of Fe (62.71 ± 0.34 ppm), Mn (21.05 ± 0.18 ppm), Cu (10.12 ± 0.17 ppm) and low in Pb (6.17 ± 0.13 ppm), Ni (1.8 ± 0.56 ppm), Ca (0.41± 0.07%), Cr and Cd were not detected throughout the development stages at 20 WAA as compared to immature stages of 4, 6, 8, 10, 12, 14 and 16 WAA. The level of antinutrient factors are oxalate (5.2 ± 0.91 mg/100 g); phytate (0.32±0.02 mg/100 g) and cyanogenic glycosides (0.14 ± 0.02) which recorded lower content, except tannins (82.11 ± 0.33) mg/100 g which recorded significant ($p < 0.05$) higher content at 20 WAA when compared with immature stages in 4, 6, 8, 10, 12, 14 and 16 WAA. This study revealed the nutritional profile of the fruit pulp as good sources of plant protein, carbohydrate and fat, with a reduction in the level of some anti-nutrients in matured fruits which are potentials that could be exploited by food and pharmaceutical industries.

Key words: *Dacryodes edulis*, proximate, antinutrient, n-hexane, anthesis.

INTRODUCTION

In Africa,fruits are in high demand, and this is because they are complemented with food to ensure a balanced diet. Fruits serve as sources of fat,carbohydrate, vitamins and minerals hence, they also become important when

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important when the functions of these nutrients are being considered in the body (Olusanya, 2008). Fat and oil are used in a variety of ways, for food texturing, baking and frying, and also used industrially, in the manufacture of soap, detergent, cosmetics and oil paints. In plants, oil is deposited in the seeds mostly in the endosperm along with carbohydrates where they jointly nourish the embryo (Ajibesin et al., 2008). It is also found in some plants mesocarp (palm fruits). Nutritional and industrial processes have increased the demands for oils and this in turn has led to the search for oils from different types of seeds (Ajibesin et al., 2008).

Dacryodes edulis (also called African plum, African pear or Safou) is an indigenous fruit tree in the humid lowlands and plateau regions of West, Central African and Gulf of Guinea countries. *Dacryodes edulis* belongs to the *Burseraceae* family (Kengue and Nyagatchou, 1990; Anonymous, 2011a). It is an evergreen tree indigenous to the central Africa and Gulf of Guinea regions. The genus name is derived from the Greek word '*Dakruon*'(a tear) in reference to the resin droplets that appears on the bark surface of its species. The species-specific name *edulis* means edible (Kengue and Nyagatchou, 1990; Anonymous, 2011a). The genus *Dacryodes* comprises about 40 species, occurring in the American, Asian and African tropics. In Africa, about 20 species have been described (Anonymous, 2011b). In Southeast Nigeria, the trees are grown around homesteads and flowering takes place from January to April. The major fruiting season is between May and October (Kengue and Nyagatchou, 1990). The role of fruits to a healthy and nutritious diet, the world over is a well-established fact. *D. edulis* is a tree cultivated widely for its edible and nutritious fruits. Generally, the fruit may be cooked in hot water, or roasted/baked in an oven at about 50°C. The cooked fruit can be eaten with maize, plantain, cassava, cocoyam, bread, etc.

The entire plant of *D. edulis* has pharmaceutical properties that are variously exploited by many African communities (Kengue, 2002). Oral treatment against leprosy and it is also gargled as mouthwash for the treatment of tonsillitis. In the western parts of Cameroon, the bark is crushed and used in concoctions against dysenteries while in central Cameroon, the bark is used to treat a toothache. The leaves are boiled in combination with *Lantana camara*, *Cymbopogon citratus* and *Persea Americana*, yielding a steam bath taken to treat fever/headaches and malaria in the Republic of Congo. The leaves made into a plaster have been recently reported to treat snake bites in South West Cameroon (Jiofack et al., 2010). The leaves are also crushed and the resultant juice used to treat skin diseases such as scabies, ringworm, rashes, while twigs from branches are sometimes used as chewing sticks (Igoli et al., 2005; Ajibesin et al., 2008; Okwu and Nnamdi, 2008). The leaves and seed are used in Nigeria for animal feed (Ajibesin et al., 2008). The resin from the bark has long been reported to treat parasitic skin diseases and jiggers in Nigeria, whereas when applied in lotions and body creams it smoothens the skin. The resin is also used in some communities as incense and is believed to send off evil spirits in Nigeria (Sofowora, 2008). *D. edulis* is one of the tropical trees whose fruits contain oils in its pulp and seed kernel. The pulp which is commonly eaten raw or cooked; it is also usually processed for the constituent oil which is popularly referred to as *atile* oil in some parts of Nigeria, using cold-pressed method of oil extraction. The properties and qualities of the oil have been investigated to some extent (Danjouma et al., 2006) and the oils have been shown to have potential industrial uses in production of pharmaceutical and personal products and, as a thermal fluid among others (Ajiwe et al., 1998). However, unlike some other oil-bearing materials such as groundnut, soyabean, palm pulp and palm kernel, the extraction of oils from elemi pulp and kernel are not being carried out at commercial level at present, despite ready availability of the fruit in large quantity in Nigeria and elsewhere in sub-Sahara Africa. This situation would improve if information on the composition and safety consumption of the pulp oil are available. Hence, this present study investigated the proximate, antinutritive and mineral composition of *D. edulis* fruit pulp at different stages of fruit development.

MATERIALS AND METHODS

Matured fruits of *D. edulis* were collected from private farmland in Ondo Town, Ondo State, Nigeria. The fruits were authenticated by the Department of Botany, University of Medical Sciences, Ondo. A voucher specimen of each plant was thereafter deposited in the herbarium of the same department. The plant grows in tropical climate and flower at the beginning of the rainy season (January – April) and bears fruit during 2-5 months after flowering (May – October).

Preparation of sample

Forty fruits were collected randomly (to provide enough fruits for oil extraction) from each of the studied trees (of between 4-8 years old) at biweekly intervals starting from the fourth week after fruit set until senescence. The collected fruits which were of variable sizes (4 to 12cm), dark-blue to violet in color and the pulp were 3.5 to 9.0mm thick, were cleaned with a moist soft cotton wool and then the seeds were carefully separated from the fruits. Part of the separated nuts was immediately used for oil extraction, while the remaining part was dried at 65°C for 4 h in an oven, crushed with a laboratory mortar and pestle and kept in a well-labelled airtight polyethylene bags or screw-capped bottles at 4°C for subsequent biochemical analysis.

All reagents used were of analytical grade purchased from Sigma Chemicals Co, London, and BDH Chemicals Ltd., England.

Extraction of oil

The soxhlet extraction method was employed. The sample (5 g)

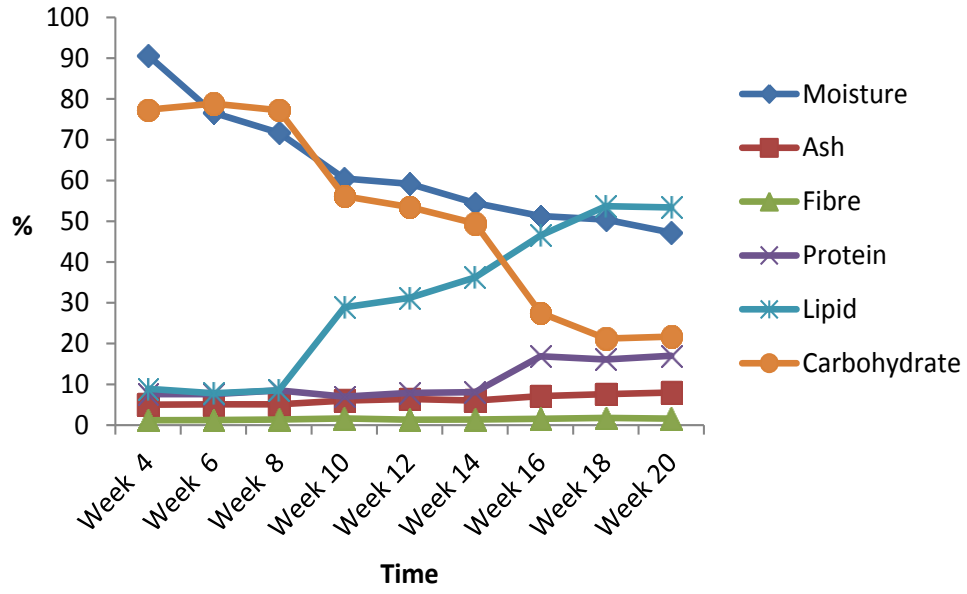

Figure 1. Proximate composition of *D. edulis* 4-20 WAA of fruits development. Values are mean ± SEM (* = P<0.05).

was weighed into a weighed filter paper and folded neatly. This was placed inside the pre-weighed thimble (W_1). The thimble with the sample (W_2) was inserted into the soxhlet apparatus and extraction under reflux was carried out with the n-hexane (40-60°C boiling range) for 6 h. At the end of extraction, the thimble was dried in the oven for about 30 min at 100°C to evaporate off the solvent and was cooled in a desiccator and later weighed (W_3). The percentage fat extracted from a given quantity of sample was then calculated.

Calculations

% Crude fat (W/W) = [LOSS IN WEIGHT SAMPLE [($W_3 – W_1$) / original weight of sample ($W_2 – W_1$)] x 100

Proximate composition analysis

The proximate compositions of the wet and dry samples are analyzed for the moisture content, carbohydrate, crude lipids, protein, ash and crude fiber by the methods of the Association of Official Analytical Chemists (AOAC, 1990).

Antinutrient screening

Quantitative phytochemical analyses of anti-nutrients were determined using the methods of Sofowora (1993). The mineral content was according to methods as described by AOAC (1990). All determinations were done in duplicates.

Statistical analysis

All the experiments were done in triplicates. The mean and standard deviations were reported. Data were subjected to analysis of variance (ANOVA). Significance of mean difference was determined using least significant difference (LSD).

RESULTS

The proximate composition of *D. edulis* fruits are shown in Figure 1. The *D. edulis* fruits were found to have a higher amount of lipids, carbohydrate and ash content at the matured stage of fruits development (Table 1).

The levels of iron (Fe), manganese (Mn), chromium (Cr), nickel (Ni), lead (Pb), cadmium (Cd) and copper (Cu) in *D. edulis* are shown in Figure 3. The results show that Fe and Mn have the highest levels, while Cr and Cd were not detected from immature to matured fruits (Table 2).

The results (Figure 2) reveal that the levels of phytate, oxalate and hydrocyanic acid were very low in concentration and tannin was higher when compared with the other anti-nutritional factors assessed (Table 3).

DISCUSSION

Determination of the proximate composition of plants is important because it predicts the profitability of a given plant as potential source of nutrients. High oil content in plant seeds implies that processing them for oil would be economical (Ikhuoria et al., 2008). The oil yield found in the studied plants compares favorably well with the oil yields reported for some commercial plant oils such as

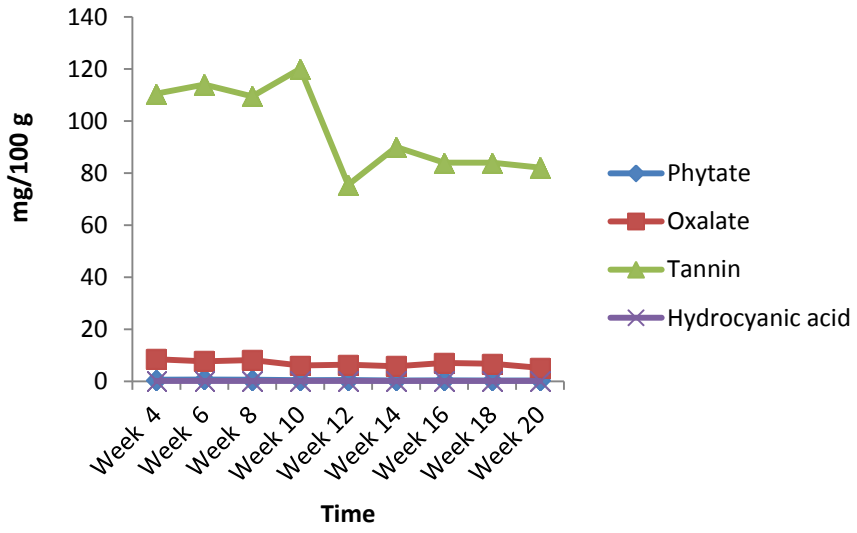

Figure 2. The anti-nutritional content of *D. edulis* 4-20 WAA of fruits development. Values are mean ± SEM (* = P<0.05).

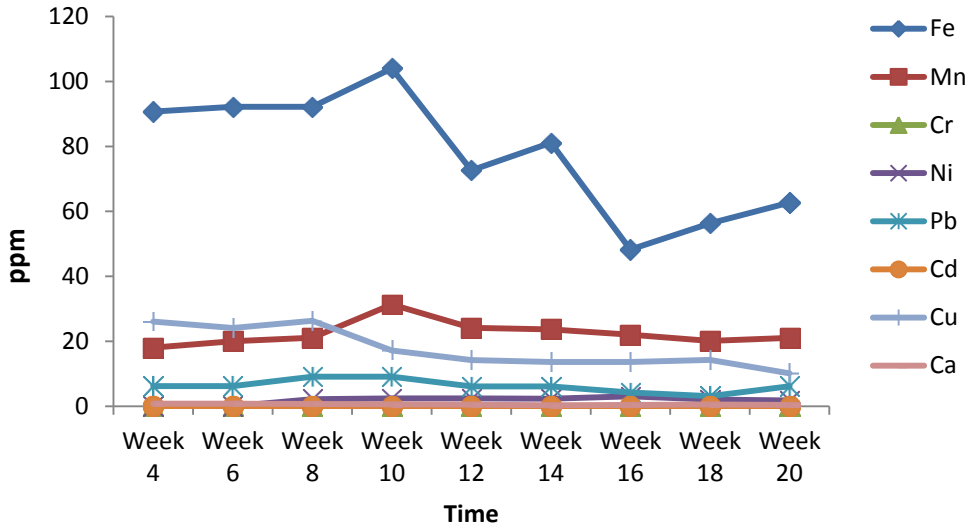

Figure 3. The elemental content of *D. edulis* 4-20 WAA of fruits Development. Values are mean ± SEM (* = P<0.05).

cottonseed (36%), groundnut (40%), oil palm (22%) and corn oil (3.4%) (Rossel, 1987; Edem et al., 2009).

The fat content (within 4th and 10th WAA) showed slight variation but increased rapidly as the week increases for both plants. This suggests a slow formation of the chemical constituents during the fruit development (Nwosuagwu et al., 2009). An increase in the fat content from the (12th – 20th WAA) showed full maturity. This agreed with earlier findings by Nwosuagwu et al. (2009) and Fonteh et al. (2005), which correspond to the period of significant external color change (blue-black). This results supports the fact that *D. edulis* pulp are relatively richer source of lipids than other conventional sources such as soybean (17.0 to 20.0%), oil palm (20 to 22%) and cotton seed (28 to 32%), and can replace some in culinary uses (Mbofung et al., 2002).

The moisture content (on a wet weight basis) of *D. edulis* fruit pulp decreases with maturation and was consistent with earlier studies (Nwosuagwu et al., 2009; Nwaoguikpe et al., 2012). The decrease in the moisture

Table 1. Proximate composition of the *D. edulis* at different stages of fruit development.

Week	Moisture content (%)	Ash content (%)	Fibre Content (%)	Protein content (%)	Lipid content (%)	Carbohydrate content (%)
4	90.7 ± 2.04	5.0 ± 0.009	1.22 ± 1.29	7.6 ± 0.91	8.9 ± 2.01	77. 38 ± 0.93
6	76.6 ± 1.10	5.1 ± 0.13	1.26 ± 0.68	7.5 ± 1.70	7.8 ± 1.88	78.84 ± 0.86
8	71.7 ± 0.93	5.1 ± 0.94	1.36 ± 0.39	8.5 ± 1.34	8.5 ± 1.13	77.24 ± 1.74
10	60.5 ± 0.99	6.0 ± 0.31	1.64 ± 0.82	7.0 ± 2.11	28.9 ± 0.99	56.16 ± 1.81
12	59.2 ± 1.8	6.4 ± 1.01	1.32 ± 0.86	7.9 ± 0.79	31.2 ± 1.71	53.47 ± 2.35
14	54.5 ± 0.46	6.0 ± 0.38	1.39 ± 0.79	8.1 ± 0.09	36.2 ± 2.81	49.51 ± 1.81
16	51.3 ± 1.31	7.1 ± 0.61	1.56 ± 0.23	16.9 ± 0.86	46.5 ± 0.57	27.54 ± 1.36
18	50.4 ± 0.84	7.6 ± 0.37	1.76 ± 0.44	16.1 ± 1.33	53.7 ± 1.76	21.24 ± 2.04
20	47.2 ± 1.20	8.0 ± 0.81	1.60 ± 0.75	17.0 ± 0.48	53.4 ± 1.35	21.70 ± 0.98

Data are the average of 3 replicates ± SE

Table 2. Elemental composition of the *Dacryodes edulis* pulp at different stages of development.

Week	Fe (ppm)	Mn (ppm)	Cr (ppm)	Ni (ppm)	Pb (ppm)	Cd (ppm)	Cu (ppm)	Ca (%)
4	90.71 ± 0.13	18.04 ± 0.34	Nd	Nd	6.18 ± 0.08	Nd	26.07 ± 1.21	0.809 ± 0.09
6	92.19 ± 0.05	20.04 ± 0.04	Nd	Nd	6.22 ± 0.13	Nd	24.08 ± 0.31	0.804 ±0.11
8	92.07 ± 0.47	21.08 ± 0.10	Nd	2.17 ± 0.17	9.11 ± 0.48	Nd	26.31 ±0.81	0.685 ±0.08
10	104.0 ± 0.34	31.32 ± 0.44	Nd	2.41 ± 0.4	9.13 ± 0.06	Nd	17.17 ± 1.21	0.601 ± 0.04
12	72.61 ± 0.03	24.14 ± 0.08	nd	2.41 ± 0.4	6.08 ± 0.04	Nd	14.24 ±0.31	0.50 ± 0.09
14	81.11± 0.41	23.64 ± 0.14	Nd	2.33 ± 0.56	6.08 ± 0.02	nd	13.62 ± 0.43	0.314 ± 0.01
16	48. 22± 0.07	22.04 ± 0.34	nd ±	3.07 ± 0.41	4.22 ± 0.008	Nd	13.71 ± 0.31	0.424 ± 0.01
18	56.28 ± 0.81	20.11± 0.10	Nd	2.07 ± 0.07	3.08 ± 0.14	Nd	14.28 ± 1.03	0.557 ± 0.07
20	62.71 ± 0.34	21.05 ± 0.18	Nd	1.80 ± 0.56	6.17 ± 0.13	Nd	10.12 ± 0.17	0.411± 0.07

Data are the average of 3 replicates ± SE; Nd= not detected.

content and the concomitant increase in the fat content in the studied plants demonstrated a close negative trend and showed that the two constituents remained negatively constant for fruits widely differing in oil content (Bezard et al., 1991). In this study, the crude protein content of *D. edulis* extracts (within 4 -20 WAA) significantly increased ($p< 0.05$).

The crude protein value at 20 WAA agrees with the results of Nwosuagwu et al. (2009) and Kinkela et al. (2006). In contrast, it is higher than the range values (24.0 to 60.0%) reported by the authors (Ayuk et al., 1999; Mbofung et al., 2002). The variation in the present study and those reported by earlier studies could be attributed to the difference in the methods of analysis employed, genetic makeup and racial origin of the fruit (Silou, 1996). This equally emphasized the rich source of the fruits in plant proteins with a high content of available lysine [27.0 – 39.0 mg/100 g protein (Mbofung et al., 2002)].

The crude fiber content significantly increased ($p<0.05$) with an increase in development but had a slight variation (within 4 and 14 WAA), suggesting a period of slow formation of the indigestible carbohydrate. The fibre content within weeks 16 and 20 WAA were lower than that previously reported (Nwosuagwu et al., 2009). The state of development at the time of harvest and geographical growth location of the fruit plant could influence variation (Itoh et al., 1975).

D. edulis fruit pulp ash content showed an increase as the fruit increase in maturity, although a slight variation occurs at the early stage of maturity. This agrees with previously reported works on the plant (Fonteh et al., 2005). The carbohydrate content decreased ($P<0.05$) with maturation. The metabolism of the polysaccharides in the cell starch hydrolysis which contribute to the increase in the total sugars observed in climacteric fruits could have been responsible for this decrease (Biale and Young, 1962). Also, the decrease may be attributed to the changes in the quantity of cell wall materials during ripening (Nwosuagwu et al., 2009).

The most common antinutritional factors in fruits are oxalate, tannins, phytic acid and hydrocyanic acid

Table 3. Anti- nutritional composition of *Dacryodes edulis* pulp at different stages of fruit development.

Week	Phytate (mg/100 g)	Oxalate (mg/100 g)	Tannins (mg/100 g)	Hydrocyanic (mg/100 g)
4	0.66±0.11	8.5±0.91	110.53 ± 0.66	0.16 ± 0.04
6	0.67±0.11	7.7±0.51	114.1± 0.83	0.15 ± 0.04
8	0.61± 0.04	8.2±0.46	109.50 ± 0.16	0.15 ± 0.01
10	0.42±0.01	6.1±0.31	120.12 ± 0.6	0.17 ± 0.06
12	0.54±0.01	6.4±0.13	75.51 ± 0.87	0.13 ± 0.03
14	0.32±0.03	5.8 ± 0.84	90.03 ± 0.31	0.14 ± 0.03
16	0.36±0.08	7.1 ± 0.46	84.00 ± 0.41	0.14 ± 0.01
18	0.30±0.01	6.8 ± 0.01	84.10 ± 0.94	0.14 ± 0.02
20	0.32±0.02	5.2 ± 0.91	82.11 ± 0.33	0.14 ± 0.02

Data are the average of 3 replicates ± SE.

(Ibanga and Okon, 2009). A daily intake of 450 mg/100 g of oxalic acid has been reported to reduce the bioavailability of such metal as calcium. Phytic intake (4.00 – 9.00mg/100g) reduces iron (Fe) absorption by 4-5 folds in humans (Ibanga and Okon, 2009). The anti-nutrients compositions of *D. edulis* fruit pulp were generally low. In matured fruits (20 WAA), phytate and cyanic levels were low. These are in agreement with the results obtained from previous work (Ibanga and Ekon, 2009). But it should be noted that the concentration of anti-nutrients are reduced during processing and as such, there might be a reasonable concentration of anti-nutrients in raw fruits that make consumption of the raw fruits harmful to health. It is therefore, safe to consume the fruits when cooked or boiled (Akwaowo et al., 2000; Ibanga and Ekon, 2009). As seen in the results, the immature fruits contain higher levels of these anti-nutrients than the matured. It may be unsafe to consume immature fruits of *D. edulis.* The oxalate concentration in D. edulis was within normal range as stipulated by WHO throughout the maturation period (Udosen and Ukpana, 1993).

In the elemental study, the results reveal that the fruits of *D. edulis* did not contain chromium (Cr) and cadmium (Cd) throughout the maturation of the fruit, but traces of lead (Pb) and nickel (Ni) were detected in the studied plants which were below the maximum permissible level (Table 2). According to WHO, the permissible limit of lead is 10 ppm, cadmium 3.0 ppm and chromium 2 ppm (WHO, 1991). Iron (Fe) content was prominently higher than all the metals analyzed in the fruit, followed by manganese (Mn), copper (Cu) and calcium (Ca) in *D. edulis* fruit pulp. Cu plays an important role in the metabolism of Fe and as a cofactor in the enzymatic systems. Cu deficiencies lead to impairment of Fe absorption. In severe cases of copper deficiency, the development of anemia has been documented (James, 2009). Mn in fruits shows that *D. edulis* can be used to treat bones disease (James, 2009). On the basis of the results of this study, *D. edulis* fruit is rich sources of Fe, Cu and Mn. Hence, this fruit having these trace elements are helpful in maintaining various functions of human body.

The presence of heavy metals in fruits can cause serious diseases to the consumers. Pb causes adverse effects on physiological and behavioral activities in living beings. Its chronic toxicity causes kidney dysfunction, osteomalacia and obstructive lung disease (IARC, 1994). Cadmium is another carcinogen associated with the risk of serious health hazard (IARC, 1994). Liver and kidney are considered as the main target organs in acute and chronic Cd exposure (Asagba and Obi, 2003). On the basis of the low heavy metal content of the studied plant, this makes the fruit pulp safe for consumption which is in agreement with previous findings.

Conclusion

D. edulis pulp oil is consumed to a limited extent in Nigeria but of which no large-scale use is off, partly because there is little information on their nutritive values. These oilseeds can be exploited as sources of edible and industrial oils. Furthermore, this study has shown that the pulp oil is comparable to other currently used vegetable oils, and has satisfactory nutritional value.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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