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Table of Content

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Full Length Research Paper

Physicochemical and microbiological stability of muffins packed in actives edible coatings from cassava starch: Inverted sugar/sucrose and natural additives

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The aim of this study was to develop edible coatings containing natural additives incorporated to the matrix of cassava starch, for use in muffins, as well as to evaluate the efficacy of its use in the increase of shelf life and maintenance of the products characteristics. Seventeen film forming formulations were developed following a central composite experimental design (CCD). The influence of independent variables (soluble coffee, cocoa powder and propolis extract) was determined on the sensorial (colour, taste, texture and dissolution) and physical-chemical parameters (water activity, humidity, hardness, chewiness, cohesiveness and elasticity) of the muffins with edible active coatings. Two new formulations were selected for the study of microbiological and physical-chemical stability during the storage. In the sensorial evaluation, it was verified that the variables studied exerted a significant effect ($p<0.05$) for colour, taste and solubility of the coated product. Regarding the texture, none of the variables showed a significant effect ($p>0.05$). For colour, the concentrations of soluble coffee and cocoa powder were significantly influenced ($p<0.05$) both by the tasters and by instrument (L*, a*, and b*). In the shelf life test, the active coating containing soluble coffee (0.76%), cocoa powder (0.22%) and propolis extract (0.82%) increased the shelf life of the muffins in up to six times, when compared with the control. The result was approximately 87 days shelf life in normal storage conditions (25°C). It was evidenced that the additives tested have an antimicrobial action, associated to the preservation of the other stability properties of the product.

Key words: Additives, stability, shelf life, edible coatings.

INTRODUCTION

Cakes are confectionary products much appreciated as desert or snacks, coming in different formats, tastes and textures, which vary with formulation or the method used in manufacturing (Jan et al., 2018). It is a product created through the mixture, homogenization and convenient cooking of the dough prepared with fermented or non-

fermented flours and other food items such as milk, eggs and fat (Gutkoski et al., 2009). A strong tendency of the industrialized cakes market is the snacks, small cakes wrapped in individual packaging, also called muffins (Channaiah et al., 2017; Kaur and Kaur, 2018). It is noted that one of the main factors associated with poor quality in cakes is the low specific volume and the non-uniform structure of the centre, besides hardness, loss of humidity and microbial development during shelf life (Abdou et al., 2008; Liu et al., 2018).

The minimization of humidity migration during storage could be reached with the use of films and coatings, giving special attention to edible ones (Fakhouri et al., 2015; Ollé Resa et al., 2016). Edible coating could be defined as a fine layer of biodegradable material, deposited on a food item. Its purpose is to inhibit or minimize the migration of humidity, oxygen, carbon dioxide and aromas, working as a semi-permeable barrier and flavouring, antioxidants and antimicrobial carrier, promoting the improvement of the product's texture and colouring and the increase of the products shelf life (Razavi et al., 2015; Antoniou et al., 2015; Treviño-Garza et al., 2015; Galus and Kadzińska, 2015; Pagno et al., 2016).

Novel food packaging technologies arose as a result of consumer's desire for convenient, ready to eat, tasty and mild processed food products with extended shelf life and maintained quality (Majid et al., 2018). However, currently, researchers have been exploring novel and reliable alternatives in order to delay bacterial growth and also contribute to preserving the freshness and quality of food products (Tian et al., 2018). Edible films are an example of these new products preservation methods. Edible coatings have been manufactured with incorporated antimicrobial and antioxidant agents, which can reduce spoilage events by enhancing the shelf life of food products (Ramírez-Guerra et al., 2018; Resende et al., 2018).

The biopolymers mostly used in the manufacturing of these coatings are the proteins (gelatines, casein, egg albumin, wheat gluten, zein and myofibrillar proteins), the polysaccharides (starch and its derivatives, pectin, cellulose and its derivatives, alginate and carrageenan) and the lipids (acetylated glycerides, stearic acid, wax and fatty acid ester) or their combination (Oriani et al., 2014; Antoniou et al., 2015; Gutiérrez et al., 2015; Ban et al., 2015; Barba et al., 2015; Aydogdu et al., 2018).

The interest of the food industry on edible films and coatings for the control of humidity transference in food is justified by the need to maintain the quality throughout the whole shelf life of the product. Besides, there is also a need to decrease the volume of disposed synthetic packaging (Vilela et al., 2018; Vital et al., 2018; Rangel-Marrón et al., 2019). Another aspect is the possibility of introducing additives to films and coatings, such as antioxidants (Ganiari et al., 2017), aromas and antimicrobial agents, improving the product's integrity (Sun et al., 2014; Gutiérrez et al., 2016).

Due to its availability, biodegradability, renewability, film-forming ability, and low cost, starch from different botanical sources (cassava, corn, wheat, rice, potato, pea, and others) is one of the most promising natural polymers for packaging applications (Sapper et al., 2019). Studies have shown that the use of cassava starch as raw material to manufacture edible films and coatings provides a good aspect and an intense shine, making the food items more commercially attractive due to the more resistant, transparent and efficient biodegradable packaging, acting as barriers against water loss (Farris et al., 2014; Da Silva et al., 2015; Abreu et al., 2015). Due to the food grade of the cassava starch film, it can be ingested as a whole packaged product (Veiga-Santos et al., 2007; Fakhouri et al., 2015; Gutiérrez et al., 2016). In addition, there is the concern of consumers on food safety, increasing the search for natural additives with antimicrobial action (Bajpai et al., 2018; Karmaus et al., 2018), among other functions, to be used in substitution to the synthetic additives normally used with this end in bakery, such as potassium sorbate, citric acid and calcium propionate (Valerini et al., 2018). The sources of bioactive compounds are cocoa powder, coffee and propolis extract, which can be used as sources of natural antimicrobial compounds (Sorour et al., 2014; Femi-Adepoju and Olufemi Adepoju, 2014; Bonilla and Sobral, 2016).

In this context, the aim of this study was to develop edible coatings containing different natural additives (soluble coffee, cocoa powder and propolis extract) incorporated to the matrix of cassava starch, plasticised with sucrose and inverted sugar for use in muffins, as well as to evaluate the efficacy of its use in the increase of shelf life and maintenance of the products characteristics.

MATERIALS AND METHODS

Raw material

In the development of edible active coatings, cassava starch (Cargill Agrícola S/A), inverted sugar (Guarany S/A), sucrose (União, Brasil), cocoa powder (Garoto, Brasil), soluble coffee (Nescafé – Brasil), propolis extract (Prodapys, Brasil) and BOPP (Bioriented Polypropylene) metallic packaging (Doces Sabor da Bahia, Brasil) were used. To prepare the muffins, products were used as a wheat flour, refined sugar, pasteurized liquid eggs, liquid milk, lectin and glycerine P.A., from the local market in Salvador, Bahia, Brazil.

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Table 1. Actual values of each natural additive (independent variables) for the seventeen formulations (active edible coatings) studied.

Formulation	Independent variables		
	Soluble coffee (X1)	Cocoa powder (X2)	Propolis extract (X3)
F1	0.40	0.40	0.40
F2	0.40	0.40	1.60
F3	0.40	1.60	0.40
F4	0.40	1.60	1.60
F5	1.60	0.40	0.40
F6	1.60	0.40	1.60
F7	1.60	1.60	0.40
F8	1.60	1.60	1.60
F9	0.00	1.00	1.00
F10	2.00	1.00	1.00
F11	1.00	0.00	1.00
F12	1.00	2.00	1.00
F13	1.00	1.00	0.00
F14	1.00	1.00	2.00
F15*	1.00	1.00	1.00
F16*	1.00	1.00	1.00
F17*	1.00	1.00	1.00

*Central points.

Experimental planning

Seventeen film forming formulations were developed following a central composite experimental design (CCD), with an order model 2^3, containing 4 axial points, 10 orthogonal points and three central points (Table 1). The solutions were applied as edible coatings for muffins, and evaluated alongside the control (without coating).

With the results of the parameters of muffins coated with the seventeen formulations, Pareto graphs were built to determine the influence of independent variables (soluble coffee, cocoa powder and propolis extract) on the sensorial (colour, taste, texture and dissolution) and physical-chemical parameters (water activity, humidity, hardness, chewiness, cohesiveness and elasticity) of the muffins with edible active coatings, for selection of two new formulations. The results that showed a significant influence on the Pareto graphs were evaluated through the ANOVA test, at a confidence level of 95%. Two new formulations were selected for the study of microbiological and physical-chemical stability during the storage, as subsequently shown.

Preparation of films solutions and application of edible active coatings on muffins

A solution of cassava starch (5.0%), inverted sugar (1.4%), sucrose (0.7%) and natural additives, such as soluble coffee (0.0-2.0%), cocoa powder (0.0-2.0%) and propolis extract (0.0-2.0%) (Table 1), dissolved in distilled water, warmed at 70°C under constant agitation and cooled at room temperature (25±2°C) was prepared for the formulations of the film forming solutions. Afterwards, the solutions were applied in two layers in the muffins samples, using a silicon brush. After the application, the muffins were put in the oven at 190 to 200°C temperature for 5 min, to completely dry the coating (Figure 1).

Characterization of muffins with edible active coatings sensorial evaluation

After coating, samples of all formulations were submitted to a sensorial evaluation of reaction to colour, taste, texture and dissolution. In order to determine the scores given to the products, a hedonic scale structured in nine points and a team of 60 tasters were used (IAL - Instituto Adolfo Lutz, 2008).

Water activity (Wa) and humidity (RH)

The Wa was determined as a decagon (Lab Master, Novasina - TECNAL, SP/Brazil), with an electrolytic measurement cell CM-2. The samples were pre-stored at 60% RH at 25°C (Veiga-Santos et al., 2005). The humidity content (%) was determined by infrared drying (Mettler LTJ) (IAL - Instituto Adolfo Lutz, 2008).

Instrumental analysis of texture and colour

The hardness, cohesiveness, elasticity and chewiness were evaluated on the texture analyser CT3 Texture Analyzer (Brookfield), according to method AACC 74-09 (American Association of Cereal Chemists, 2000). The colour was determined on Konica Minolta colorimeter - TECNAL, through the following parameters: L* (luminosity), a* and b* (chromaticity coordinates). In this system, L* indicates luminosity (0 = black and 100 = white) and

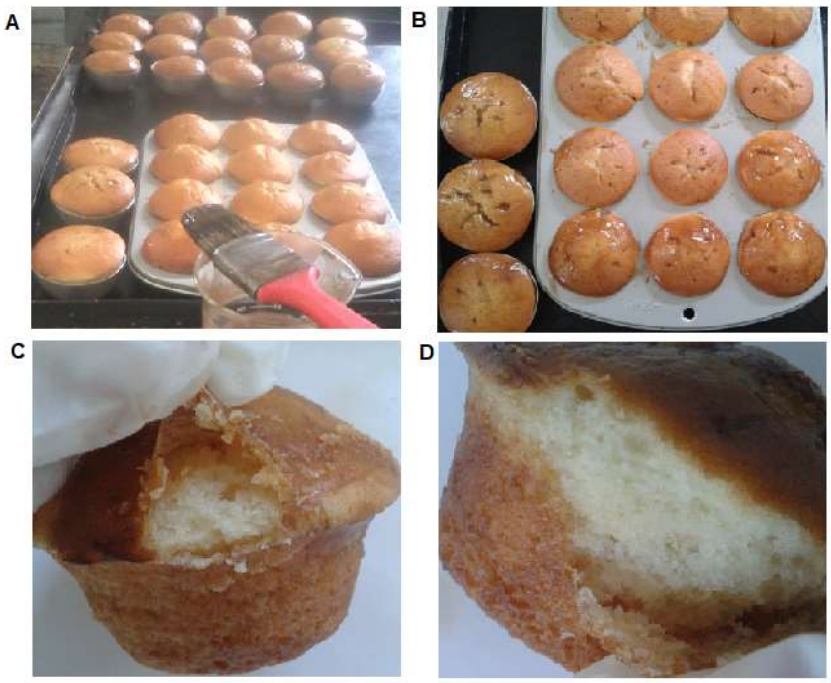

Figure 1. Application of edible films on muffins (A), coating completely dry (B), and muffins with edible films (C and D).

a* and b* indicate the directions that the colour could assume (+a* = red and -a* = green; +b* = yellow and -b* = blue) (Lopes, 2005).

Microbiological analysis

The mould and yeast counts were evaluated in the muffins during storage through the surface plating method using the Agar Dicloran Rose Bengal Chloramphenicol (DRBC) medium (King et al., 1979).

Monitoring of muffins stability with edible active coatings selected during storage

The best concentrations defined by the central composite experimental design for the independent variables (soluble coffee, cocoa powder and propolis extract), in the evaluation of sensorial attributes of colour, taste, texture and dissolution were used on the development of two new formulations selected for coating the muffins.

The muffins with active edible coatings and the control (without coating), stored in a bio-oriented polypropylene (BOPP) commercial packaging were monitored in each 8 days for 48 days of storage in climatic chamber (TECNAL) on accelerated conditions of oxidation (60% UR, 35°C), by analysing Wa, humidity, texture, colour and microbiological evaluation.

Shelf life

The Arrhenius mathematical model was used to determine the reaction rate constant (K), activation energy (Ea) and temperature acceleration factor (Q10), in order to determine the shelf life of the products in normal and accelerated conditions. Besides the storage in accelerated conditions, the muffins were stored in room temperature (25°C) with Wa and microbiological evaluation performed at each eight days.

The reaction rate constant (K) was calculated using Equation 1. A graphic representation of the Wa values versus storage period for temperatures 25 and 35°C produced a linear regression equation, which enabled the determination of K in the temperatures of 25 and 35°C (K25 and K35).

$$K_T = e^{\ln A - \frac{Ea}{R}\cdot\frac{1}{T}} \quad (1)$$

For the calculation of the temperature acceleration factor (Q10), Equation 2 was used, which is multiplied by the shelf life of the muffins in accelerated conditions, determining the shelf life of these products in real conditions.

$$Q_{10} = \frac{K_T}{K_{T-10}} \quad (2)$$

where Ea = activation energy in $cal.mL^{-1}$, R = universal constant for gases (1.987 $cal.mL^{-1}K^{-1}$), T = temperature in absolute scale (Kelvin). KT = reaction rate coefficient in a determined temperature, and KT-10 = reaction rate coefficient in 10°C lower temperature.

RESULTS AND DISCUSSION

Sensorial evaluation

Edible films and coatings are innovations within biodegradable active packaging concept, which can

Table 2. Average values found for the acceptance test (Hedonic Scale) for the 17 formulations of muffins with active edible coatings and the control (without coating).

Formulation	Colour	Flavour	Texture	Dissolution
C	8.10	7.97	8.00	8.13
F1	6.83	7.40	7.13	6.30
F2	6.20	5.03	6.90	6.40
F3	4.57	6.70	6.67	6.73
F4	6.27	6.03	5.87	6.33
F5	6.77	6.70	6.70	6.83
F6	6.17	6.07	5.87	6.17
F7	4.77	5.87	5.83	5.73
F8	4.40	4.93	5.53	5.70
F9	6.17	6.77	6.90	6.80
F10	4.93	5.47	5.67	6.23
F11	6.60	5.93	6.20	6.50
F12	4.57	6.00	5.87	5.93
F13	5.83	7.33	6.30	6.73
F14	5.63	6.37	6.27	5.70
F15*	6.50	6.63	6.57	6.73
F16*	6.67	6.70	7.27	6.80
F17*	6.53	6.90	6.67	6.47

*Central points; Muffins without coating (Control, C); Muffins coated with formulations of different composition (F).

improve safety and/or functional or sensory properties while maintaining the quality of food packaging (Parra et al., 2004; Chinma et al., 2014). The average values of the sensorial parameters colour, taste, texture and dissolution of the muffins coated with the 17 formulations are shown in Table 2. The control treatment resulted in parameters with better acceptance by the tasters, understanding, at first, that the natural additives (soluble coffee, cocoa powder and propolis extract) interfere in order to reduce the acceptance of the muffins.

In the sensorial evaluation of the attribute colour, when compared with the control, only the average scores for the muffins coated with formulations F3 and F12 were less accepted by the tasters. The statistical analysis presented that the results obtained showed a significant negative effect ($p<0.05$), indicating that the increase in the concentration of variables (soluble coffee, cocoa powder and propolis extract) results in the decrease of these attributes average, which was corroborated by the surface response graphs obtained (Figure 2A and B).

The average values obtained for the attribute taste of coated muffins are adjusted to the model, and can represent the effect of the concentration of the independent variables (soluble coffee, cocoa powder and propolis extract) in a significant ($p<0.05$) and predictive manner. The response surfaces obtained for this parameter allow the visualization of the results for the sensorial analysis of attribute taste (Figure 2C, D and E). Among the tested variables, propolis contributes more significantly for decrease of the attribute taste (Figure 2D and E).

The averages reached in the sensorial evaluation of the attribute colour of the muffins with active edible coatings resulted in a low correlation with the attribute taste (R^2 = 0.38). These results mean that only 38% of the formulations with the best evaluations for colour tend to show the best evaluation for the attribute taste. For the texture evaluation, the formulations F1 and F16 obtained the higher average in the acceptance (Table 2). The statistical analysis shows that the tested variables did not exert an effect with significant difference ($p>0.05$) on the parameter. This indicates that the muffins were not altered for this attribute, independently of the concentrations tested on the coatings (soluble coffee, cocoa powder and propolis extract), which indicate a relative robustness in the formulations (Table 2 and Figure 2C).

In the sensorial evaluation of the attribute dissolution, it was noted that, in the same way identified for taste, the variable propolis extract exerts a stronger significant effect ($p<0.05$) on the decrease of this parameter (Figure 2D). Through the response surfaces (Figure 2F, G and H), it can be seen that the variables cocoa powder and soluble coffee compensate this lower acceptance even more pronounced in relation to dissolution then on the taste attribute. That way, the increase in concentration of propolis extract, as well as the interaction variables, results in a lower dissolution of the product during the

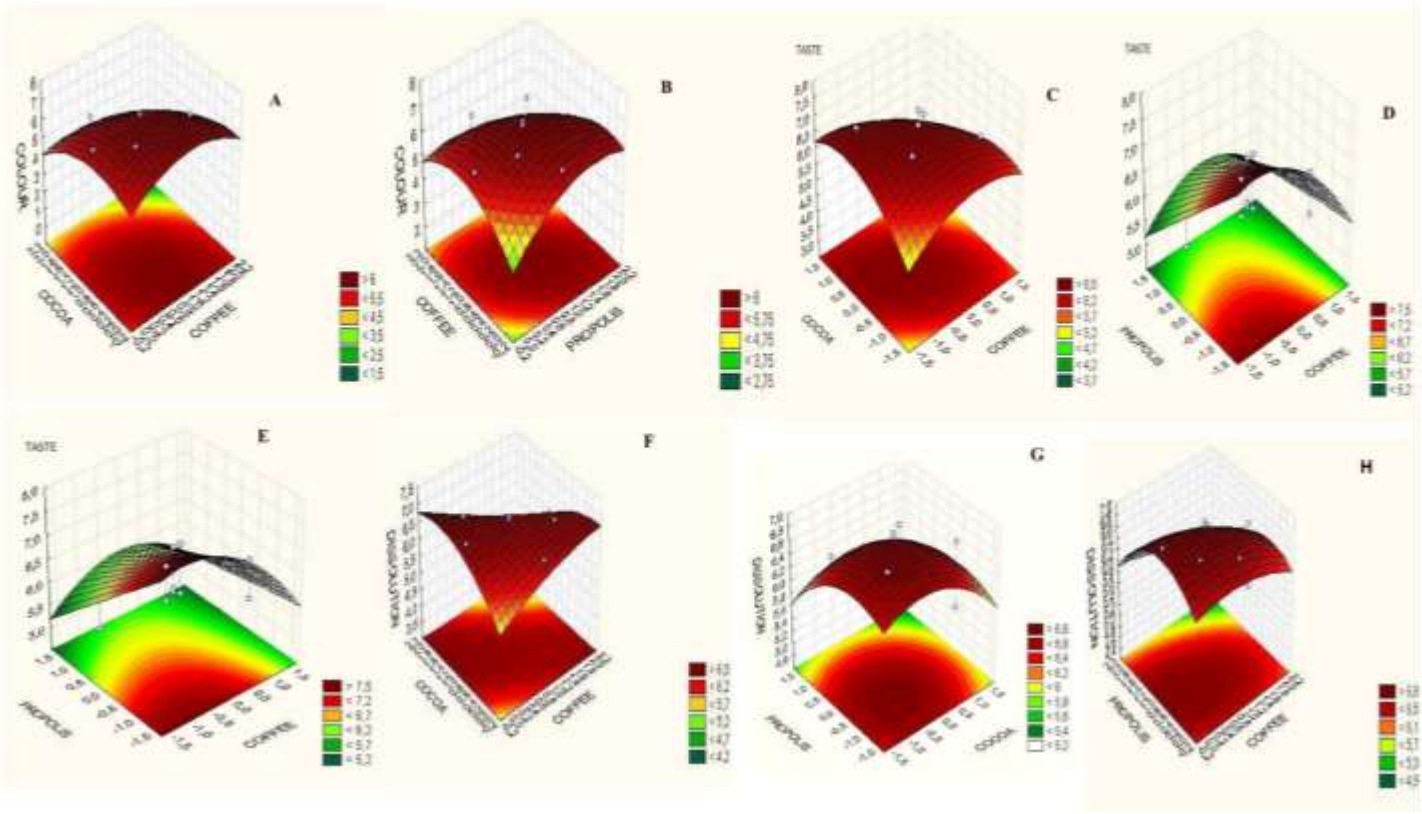

Figure 2. Response surface for sensorial analysis of the colour (A, B), taste (C, D, E) and dissolution (F, G, H) attributes of the muffins with active edible coatings, compared to the concentration of the independent variables (soluble coffee, cocoa powder and propolis extract).

muffins during chewing. This undesirable effect can be noted in the averages evaluation of the attribute dissolution, where all muffins samples coated with the 17 formulations obtained inferior marks when compared with the control (without coating) (Table 2).

These results are in accordance with the observations reported in the literature. For example, chitosan has also been widely used as an edible coating for extending the shelf life of foods, and chitosan was applied to Cavendish banana (Suseno et al., 2014). Sensory analyses were conducted to monitor the changes in color, texture, and aroma. The uncoated bananas showed unsatisfactory appearance after four days of storage, whereas the coated bananas still demonstrated an acceptable appearance up to seven day storage. The results showed that coated banana fruit demonstrated delayed ripening processes compared to the uncoated banana.

In this context, from the results of the sensorial evaluation it was possible to verify that the different concentrations, especially of the variable propolis extract, show a significant influence on the attributes taste, colour and texture, but not on the texture of the muffins. The results of the variance analysis (ANOVA) demonstrate the possibility of a significant representation of the effects of the independent variables (soluble coffee, cocoa powder and propolis extract) on the evaluated attributes.

Physical-chemical characterization of the muffins packed with edible active coatings

Table 3 shows the results of the averages for the physical-chemical characterization (Wa, humidity and texture) of the muffins covered with edible active coatings and the control.

The values of Wa of the different formulations of muffins with edible active coatings varied between 0.620 and 0.710, being therefore considered products of intermediate humidity. Comparing with the control (Wa = 0.700), it is noted that, generally, the incorporation of active compounds to the coatings contribute to the decrease in Wa of the products.

The formulation F10 containing soluble coffee, cocoa powder and propolis extract showed the lower result for Wa (0.620), associated to a lower humidity percentage (9.88%). The humidity values varied from 9.88 to 12.16% (Table 3). In the comparison between the samples of the averages by the Tukey test at 95% confidence level, for both Aw and humidity, only the sample F10 showed a significant difference ($p<0.05$) among the formulations. The statistical analysis showed that these two parameters are not significantly different ($p>0.05$) of the independent variables in the tested concentrations. The hardness values of the muffins with the different edible

Table 3. Average values (± standard deviation) for the Wa content, humidity and texture of the muffins with edible active coatings and control (without coating).

Formulation	Wa	Humidity (%)	Texture			
			Hardness (g)	Cohesiveness	Elasticity (mm)	Chewiness (mJ)
C	0.700±0.01^{ab}	12.47±0.53^{a}	227.63±404.3^{e}	0.50±0.01^{b}	8.51±0.16^{bc}	94.43±17.12^{g}
F1	0.690±0.00^{ab}	12.16±0.32^{a}	295.33±413.6^{de}	0.51±0.00^{ab}	8.74±0.04^{abc}	129.50±18.14^{efg}
F2	0.680±0.01^{bc}	11.81±0.15^{a}	280.53±164.9^{de}	0.51±0.02^{ab}	8.78±0.08^{abc}	122.60±10.33^{fg}
F3	0.690±0.00^{ab}	11.34±0.56^{ab}	355.03±742.1^{cde}	0.54±0.03^{ab}	8.70±0.17^{abc}	165.33±42.48 ^{cdefg}
F4	0.680±0.00^{abc}	11.16±0.17^{abc}	395.70±446.5^{bcde}	0.52±0.01^{ab}	8.70±0.04^{abc}	174.80±21.94b^{cdefg}
F5	0.680±0.02^{abc}	11.78±0.17^{a}	327.33±702.9^{de}	0.55±0.02^{ab}	8.96±0.20^{ab}	155.50±28.52^{defg}
F6	0.690±0.00^{ab}	11.99±0.11^{a}	281.63±495.0^{de}	0.54±0.02^{ab}	8.51±0.37^{abc}	126.23±19.27^{fg}
F7	0.690±0.01^{ab}	11.52±0.31^{ab}	365.43±1162.7^{cde}	0.51±0.02^{ab}	8.69±0.09^{abc}	156.93±45.03^{defg}
F8	0.700±0.00^{ab}	11.59±0.21^{ab}	329.06±884.8^{de}	0.52±0.01^{ab}	8.73±0.14^{abc}	146.50±40.09^{defg}
F9	0.700±0.01^{ab}	11.84±0.43^{a}	392.40±346.7^{bcde}	0.60±0.11^{a}	9.05±0.18^{a}	209.40±27.82^{bcdef}
F10	0.620±0.00^{d}	9.88±0.22^{c}	1053.33±592.0^{a}	0.47±0.02^{b}	8.39±0.09^{c}	410.43±29.90^{a}
F11	0.650±0.00^{c}	10.79±0.46^{abc}	585.40±1323.7^{bc}	0.52±0.02^{ab}	8.80±0.10^{abc}	261.47±45.66^{bc}
F12	0.710±0.01^{ab}	12.09±0.44^{a}	375.96±795.7^{cde}	0.55±0.01^{ab}	8.87±0.18^{abc}	179.47±42.00^{cdefg}
F13	0.690±0.01^{ab}	11.54±0.64^{ab}	475.93±1231.0^{bcd}	0.55±0.02^{ab}	8.96±0.04^{abc}	230.20±54.55^{bcd}
F14	0.680±0.01^{abc}	10.31±0.32^{bc}	624.33±1236.5^{b}	0.53±0.05^{ab}	8.79±0.18^{abc}	281.43±28.30^{b}
F15*	0.690±0.01^{ab}	11.09±0.60^{abc}	475.23±748.0^{bcd}	0.55±0.03^{ab}	8.96±0.18^{abc}	226.03±32.91^{bcde}
F16*	0.710±0.01^{a}	10.87±0.79^{abc}	302.03±487.9^{de}	0.55±0.02^{ab}	8.82±0.04^{abc}	138.47±15.20^{defg}
F17*	0.700±0.01^{ab}	10.98±0.64^{abc}	388.63±586.4^{bcde}	0.54±0.02^{ab}	8.89±0.09^{abc}	182.25±23.53^{bcdefg}

*Central points; Control (C); Formulations (F); Values showing the same letter, on the same column, show significant differences ($p<0.05$) by the Tukey Test at 95% confidence.

active coatings oscillated between 280.53 and 1053.33 g (control 227.63 g), depending on the concentration of the incorporated additive soluble coffee (Table 3). The only sample that showed a significant difference ($p<0.05$) compared to control was the formulation F10 with a higher concentration of soluble coffee (2.0%). The higher hardness values were the same that showed the lower humidity levels and Wa (F10, F11 and F14), which was corroborated by the existence of moderate correlations inversely proportional between hardness and humidity ($R^2 = 0.66$) and between hardness and Wa ($R^2 = 0.69$).

According to Table 3, the values of cohesiveness of the muffins varied between 0.47 and 0.60, with no significant differences ($p>0.05$) between the 17 formulations and the control. The average values found for the parameter elasticity varied from 8.39 to 9.05 mm, whereas the control showed a value of 8.51 mm. Chewiness is a parameter of texture easily related to the sensorial analysis through trained panels (Esteller and Lannes, 2005) and can be interfered directly proportional to the hardness and cohesiveness, elasticity different from elasticity (Osawa et al., 2009). This can be verified in the evaluation of the variables correlation, where it was possible to note the existence of a directly proportional correlation of this variable with hardness ($R^2 = 0.96$), and inversely proportional with humidity and Wa ($R^2 = 0.58$ and 0.65, respectively).

The comparison of the averages for the instrumental colour indices by the Tukey test identified a significant difference ($p<0.05$) of the value for chromaticity a* on the 17 formulations and control, which can be visualized on the Pareto graphs (Figure 3), demonstrating that soluble coffee and cocoa powder significantly interfere ($p<0.05$) linearly and negatively on the colour indices of the coated muffins.

There are no significant effects ($p>0.05$) of the independent variables (soluble coffee, cocoa powder and propolis extract) on the parameters Wa, humidity, hardness, cohesiveness, elasticity and chewiness of the muffins coated with the 17 formulations, indicating that the formulations present solidity, a desirable characteristic regarding the process in relation to these parameters.

Due to the effects observed on the response surfaces relative to the evaluation of the sensorial analysis for acceptance of the attributes for colour, taste, texture and dissolution, two formulations were selected for the study of microbiological stability and physical-chemical throughout storage. The selected formulations were FS1 (0.7% of soluble coffee, 0.88% cocoa powder and 0.0% propolis extract) and FS2 (0.76% soluble coffee, 0.22%

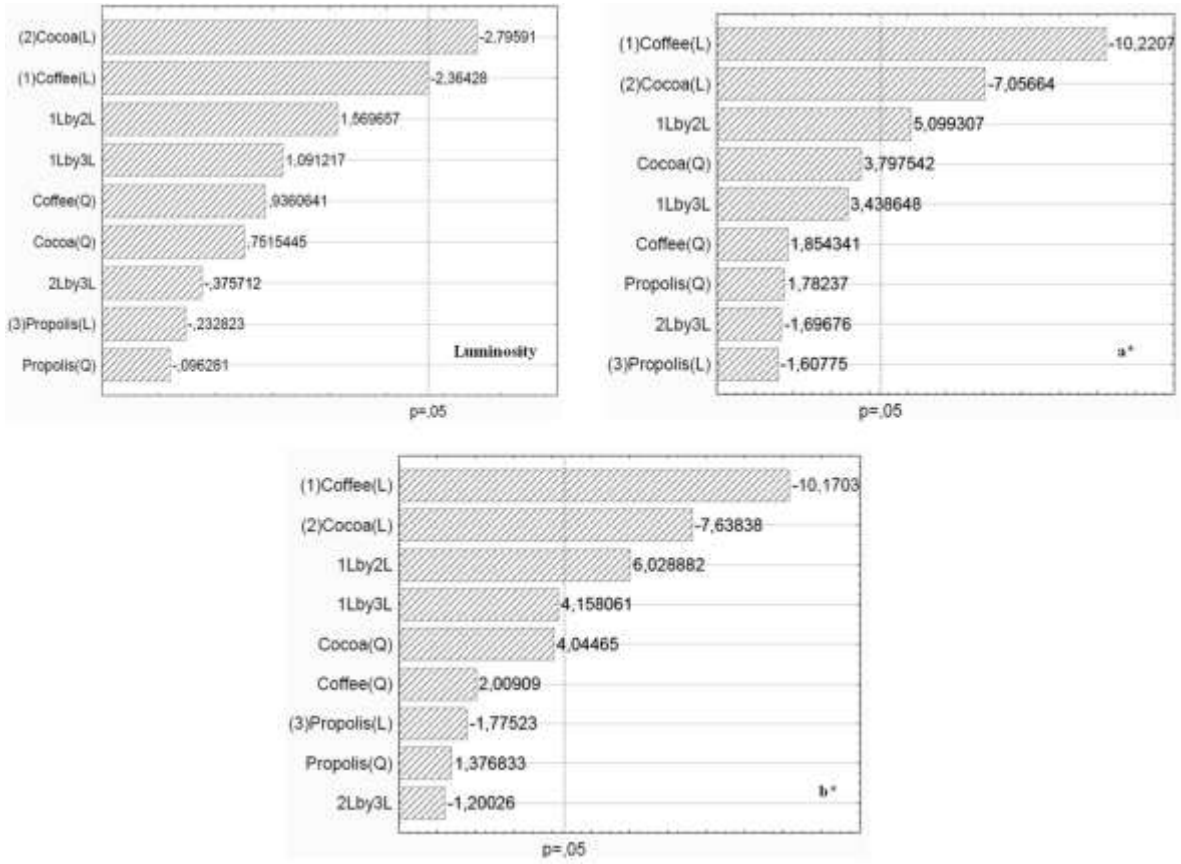

Figure 3. Pareto graph for instrumental analysis of luminosity, a* and b* of the muffins with cassava starch edible active coating (5.0%, p/p), inverted sugar (1.4%, p/p) and sucrose (0.7%, p/p), soluble coffee (0.0-2.0%, p/p), cocoa powder (0.0-2.0%, p/p) and propolis extract (0.0-2.0%, p/p).

cocoa powder and 0.82% propolis extract).

Monitoring of the microbiological and physical-chemical stability of the muffins with edible active coating selected during storage

It is important to highlight that, as the samples presented a deteriorating aspect visible to the naked eye on the coating surface, supposedly due to the growth of mould and/or yeast, they were disposed of, which occurred from 8 and 24 days, respectively, for the muffins control (no coating) and coated with the formulation (FS1). For the muffins coated with formulation FS2, this alteration was not detected until 48 days of storage, maximum limit of study, showing the most pronounced effect of propolis extract which can be strengthened by soluble coffee and cocoa powder on the coatings (Table 4). Costa et al. (2014) report a similar result on the evaluation of the antimicrobial efficacy of the active film with alcoholic extract of red propolis used to pack curdled cheese, which performed an important role in inhibiting the development of the microorganism *Staphylococcus*, proving therefore an antimicrobial effect of the active film.

Edible film coatings represent a viable preservation technology, they often constitute a barrier that delays deterioration and maintain the product structural integrity (Osorio et al., 2011). In addition, edible film coatings is a food covering film which can be eaten as part of the food, as was showed in this work. In this context, the edible active coating developed caused an inhibitory effect in the microbial growth, with an increase of at least 200% throughout the period of development of mould and yeast in accelerated conditions, when compared with muffins without coating. This represents a reduction on the losses by deterioration of the product due to the increase of its shelf life.

The contents of Wa on day zero and on the 16th day of storage shown by the muffins coated on formulations FS1 and FS2 reached a similar performance to that shown by the muffins control, with no significant difference ($p>0.05$) by the Tukey test. On the 8th day of storage, these parameters of the muffins coated by formulations FS1 and FS2 showed significant difference ($p<0.05$) in relation to the muffin control. On the 32nd day of storage it was not possible to analyse it statistically, since the

Table 4. Values of mould and yeast count of control (C) and muffins coated with formulations FS1 and FS2 throughout 48 days storage.

Days	C (UFC.g^{-1})	FS_1 (UFC.g^{-1})	FS_2 (UFC.g^{-1})
0	<10	<10	<10
8	<100	<10	<10
16	-*	<10	<10
24	-*	<100	<10
32	-*	-*	<10
40	-*	-*	<10
48	-*	-*	<10

Control (muffin with no edible active coating), FS1 = (5% cassava starch, 1,4% inverted sugar, 0,7% sucrose, 0,7% soluble coffee, 0,88% cocoa powder and 0,0% propolis extract). FS2 = (5% cassava starch, 1,4% inverted sugar, 0,7% sucrose, 0,76% soluble coffee, 0,22% cocoa powder and 0,82% propolis extract). -*Formulation presented mould visible to the naked eye.

formulation FS1 also showed a visible growth of microorganisms, and the monitoring was interrupted for this formulation. But it can be observed that the value found for the muffins coated with formulation FS2 (0.74) was similar to 16 and 24th days (0.73) (Table 5). The values found in this study are inferior to those obtained by Osawa et al. (2009) who studied the effect of edible coatings made of gelatine, stearic acid or carnauba wax on the physical-chemical properties of the chocolate cakes.

Other authors have demonstrated similar results in foods coated with edible film, because of the antimicrobial capacity of natural additives or the polymer matrix, especially against the fungi and yeast spoilage (Chiumarelli et al., 2010; Romanazzi et al., 2013). Fungal contamination on the surface of foods is a main reason that consumers do not purchase and consume these foods (fruits, breads, cheeses), and coating the surface of these foods with an edible film-forming solution can decrease this type of spoilage and increase the shelf life (Valenzuela et al., 2015).

The humidity values of the muffins coated with formulations FS1 and FS2 on day zero were similar (14.74 and 14.40%, respectively), differently from the control muffins (18.58%). So, the muffins produced with the edible active coatings (FS1 and FS2) are significantly different ($p<0.05$) of the muffins control. On the 8th day of storage, the humidity content of the muffins coated with FS1 and FS2 did not differ statistically among themselves ($p>0.05$), when compared with the value on day zero (Table 5). These results confirmed that the samples with edible active coatings are capable of interacting with the environment and with the packaged product, stopping the increase of humidity, which could damage the texture of the product, especially its smoothness.

The humidity contents on the 16th day of storage reached similar results, showing no significant difference amongst them ($p>0.05$) by the Tukey test (Table 5). During this period, a decrease in the humidity of the product coated with formulations FS1 and FS2 were observed, which could be explained by the downgrading of the starch induced by the thermal processing, causing undesirable effects such as the migration of water to the surface. These effects induce major changes in consistency and texture of the product (Martínez-Cervera et al., 2014).

On the comparison of the averages by the Tukey test at 95% confidence level, it was noted that the lower values for hardness were found for the control, which significantly differs from the muffins coated with formulations FS1 and FS2 on the 8th day of storage ($p<0.05$). The muffins coated with formulation FS2 showed less hardness between the 8 and 24th days when compared with formulation FS1 (13 to 31% lower). This effect is caused by the difference in composition of the variables on coatings (Table 5).

The hardness of the muffins coated with formulation FS1 did not differ statistically between themselves until the 16th day ($p>0.05$), but they are different statistically from the muffins with 24 days of storage ($p<0.05$, Table 4), which is approximately twice higher than the initial. Studies report double the initial hardness in cakes stored at 21°C for 21 days (Gélinas et al., 1999). An inappropriate barrier, packaging or edible coating leads to loss of humidity of the muffin. The effect of the starch downgrading should also be considered (Sluimer, 2005).

The values of hardness and chewiness of the muffins coated with formulations FS1 and FS2 progressively increased between days 0 and 24 ($R^2 = 0.84$) or 0 and 48 ($R^2 = 0.92$), respectively, whereas cohesiveness and elasticity showed a variable behaviour throughout storage.

The inversely proportional correlations between Wa and hardness of the muffins are low (R^2=0.2 FS1, R^2=0.3 FS2). However, between humidity and hardness they were higher (R^2=0.76 FS1, R^2=0.94 FS2). The same behaviour was found for the correlations between chewiness and Wa (R^2=0.49 FS1, R^2=0.26 FS2) and

Table 5. Average values (± standard deviation) Wa, humidity, hardness, cohesiveness, elasticity and chewiness of control (C) and the muffins coated with formulations FS1 and FS2 throughout the 48 days of storage.

Parameter	Days	C	FS_1	FS_2
Wa	0	0.82 ± 0.00^{aA}	0.79 ± 0.04^{Aa}	0.73 ± 0.02^{aBC}
	8	$0.83\pm0.02^{*aA}$	0.77 ± 0.01^{bAB}	0.77 ± 0.01^{bA}
	16	*	0.73 ± 0.02^{aB}	0.73 ± 0.01^{aBC}
	24	*	$0.78\pm0.00^{*aAB}$	0.73 ± 0.01^{bBC}
	32	*	*	0.74 ± 0.02^{BC}
	40	*	*	0.76 ± 0.01^{AB}
	48	*	*	0.71 ± 0.01^{C}
Humidity	0	18.58 ± 1.31^{aA}	14.74 ± 0.73^{bA}	14.40 ± 0.75^{bA}
	8	$20.37\pm0.35^{*aA}$	14.08 ± 0.69^{bA}	14.34 ± 0.28^{bA}
	16	*	13.68 ± 0.71^{aA}	13.48 ± 0.47^{aAB}
	24	*	$11.30\pm0.32^{*bB}$	13.43 ± 0.43^{aAB}
	32	*	*	12.94 ± 0.31^{B}
	40	*	*	12.52 ± 0.44^{B}
	48	*	*	10.58 ± 0.32^{C}
Hardness ($g \times 10^{-1}$)	0	145.67 ± 9.96^{aA}	344.53 ± 126.06^{aB}	385.27 ± 144.18^{aB}
	8	$139.23\pm40.60^{*bA}$	423.67 ± 16.75^{aAB}	368.90 ± 38.93^{aB}
	16	*	558.27 ± 52.04^{aAB}	444.40 ± 74.56^{aB}
	24	*	$615.17\pm137.69^{*aA}$	423.80 ± 66.37^{aB}
	32	*	*	461.77 ± 52.25^{B}
	40	*	*	527.63 ± 123.75^{B}
	48	*	*	837.77 ± 100.63^{A}
Cohesiveness	0	0.60 ± 0.00^{aA}	0.50 ± 0.10^{aA}	0.40 ± 0.10^{aA}
	8	0.60 ± 0.00^{aA}	0.60 ± 0.00^{aA}	0.60 ± 0.00^{aA}
	16	*	0.50 ± 0.00^{aA}	0.60 ± 0.00^{aA}
	24	*	0.50 ± 0.00^{aA}	0.60 ± 0.10^{aA}
	32	*	*	0.50 ± 0.00^{A}
	40	*	*	0.50 ± 0.10^{A}
	48	*	*	0.50 ± 0.00^{A}
Elasticity (mm)	0	8.50 ± 0.30^{aB}	8.80 ± 0.30^{aB}	8.80 ± 0.40^{aA}
	8	8.60 ± 0.10^{aA}	8.70 ± 0.30^{aA}	8.80 ± 0.10^{aA}
	16	*	8.90 ± 0.30^{aAB}	8.70 ± 0.10^{aA}
	24	*	8.70 ± 0.20^{aAB}	9.10 ± 0.80^{aA}
	32	*	*	8.70 ± 0.10^{A}
	40	*	*	8.80 ± 0.10^{A}
	48	*	*	8.60 ± 0.10^{A}
Chewiness (mJ)	0	68.70 ± 5.80^{aA}	138.9 ± 49.90^{aB}	142.0 ± 65.10^{aB}
	8	65.70 ± 23.60^{bA}	214.9 ± 15.70^{aAB}	181.6 ± 07.70^{aB}
	16	*	268.70 ± 39.90^{aA}	223.8 ± 39.10^{aB}
	24	*	255.20 ± 39.70^{aA}	229.0 ± 79.40^{aB}
	32	*	*	196.3 ± 21.00^{B}
	40	*	*	251.80 ± 14.60^{B}
	48	*	*	390.9 ± 6.10^{A}

Values showing different lower case or upper case letters on the same line or column, respectively, show significant differences ($p<0.05$) by the Tukey Test at 95% confidence level. C = Control (muffin without active edible coating). FS1 = (5% cassava starch; 1.4% inverted sugar; 0.7% sucrose; 0.7% soluble coffee; 0.88% cocoa powder; and 0.0% propolis extract). FS2 = (5% cassava starch; 1.4% inverted sugar; 0.7% sucrose; 0.76% soluble coffee; 0.22% cocoa powder; and 0.82% propolis extract). *Formulation that showed mould and yeast on the product surface.

Table 6. Average values (± standard deviation) of the luminosity analyses (L), a* and b* of the crust and core of the control (C) and muffins coated on formulations FS1 and FS2 throughout 48 days storage.

Color	Days	Treatments					
		Crust *Muffin*			Crumb *Muffin*		
		C	FS_1	FS_2	C	FS_1	FS_2
L*	0	51.03±0.65^{aA}	28.86±0.89^{bA}	29.76±0.26^{bA}	74.65±0.53^{aB}	70.39±0.35^{bC}	70.61±0.30^{bC}
	8	49.75±0.00^{aB}	27.69±0.83^{cA}	29.46±0.34^{bA}	80.91±0.00^{aA}	71.55±4.38^{bB}	71.56±0.53^{bB}
	16	*	28.01±0.40^{bA}	29.65±0.59^{aA}	*	72.27±0.21^{aA}	72.74±0.32^{aA}
	24	*	27.37±0.31^{bA}	28.80±0.47^{aAB}	*	72.70±0.27^{aA}	71.63±0.19^{bB}
	32	*	*	27.56±0.65^{BC}	*	*	70.27±0.23^{C}
	40	*	*	27.47±0.42^{BC}	*	*	70.56±0.37^{C}
	48	*	*	27.23±0.74^{C}	*	*	71.61±0.16^{B}
a*	0	15.66±0.00^{aA}	13.32±0.61^{bA}	13.15±0.53^{bA}	-0.45±1.74^{bA}	-2.47±3.06^{aB}	-0.17±0.02^{aD}
	8	15.29±0.98^{aA}	11.23±0.42^{bB}	12.41±0.37^{bAB}	-1.93±0.00^{bA}	0.78±0.02^{aA}	0.33±0.03^{aC}
	16	*	9.77±0.04^{bC}	12.38±0.27^{aAB}	*	1.30±0.17^{aA}	0.53±0.06^{aBC}
	24	*	7.89±0.07^{bD}	13.18±0.18^{aA}	*	3.24±0.50^{aA}	0.64±0.12^{aC}
	32	*	*	11.69±0.08^{aBC}	*	*	0.71±0.07^{BC}
	40	*	*	11.45±0.37^{BC}	*	*	1.07±0.08^{AB}
	48	*	*	11.38±0.43^{C}	*	*	1.48±0.14^{A}
bb*	0	39.04±1.07^{aA}	10.24±0.20^{cA}	12.91±0.74^{bA}	25.99±0.40^{bB}	29.25±0.16^{aB}	29.67±0.69^{aD}
	8	38.13±0.00^{aA}	9.29±1.16^{cB}	11.34±0.49^{bB}	28.79±0.00^{bA}	32.12±1.38^{aA}	31.79±0.31^{aC}
	16	*	5.95±0.29^{bC}	11.23±0.40^{aBC}	*	32.57±0.23^{aA}	32.80±0.48^{aBC}
	24	*	5.67±0.48^{bC}	11.36±0.16^{aB}	*	32.61±0.40^{aA}	32.24±0.18^{aC}
	32	*	*	10.42±0.29^{BC}	*	*	32.74±0.09^{BC}
	40	*	*	10.29±0.31^{BC}	*	*	33.94±0.60^{AB}
	48	*	*	10.14±0.15^{C}	*	*	34.48±0.36^{A}

Values showing different lower case or upper case letters on the same line or column, respectively, show significant differences ($p<0.05$) by the Tukey test at 95% confidence level. C = Control (muffin without edible active coating). FS1 = (5% cassava starch; 1.4% inverted sugar; 0.7% sucrose; 0.7% soluble coffee; 0.88% cocoa powder; and 0.0% propolis extract). FS2 = (5% cassava starch; 1.4% inverted sugar; 0.7% sucrose; 0.76% soluble coffee; 0.22% cocoa powder; and 0.82% propolis extract). *Formulation showing mould and yeast on the surface of the product.

humidity (R^2=0.43 FS1, R^2=0.91 FS2). That way, as the humidity of the muffins coated with formulations FS1 and FS2 decreased during storage (Table 5), hardness and chewiness increased.

On the analysis of cohesiveness by the Tukey test, it was possible to observe that the control muffins and those coated with formulations FS2 did not show significant difference between them ($p>0.05$) during storage. In terms of elasticity, the values varied according to the formulation of each edible coating and in face of the respective hardness values. It was noted that the samples of muffins control and coated with formulation FS1 differ statistically among themselves ($p<0.05$) throughout the storage period (Table 5). It has been reported that the value of elasticity for bakery products generally decreases during storage periods (Baik et al., 2000), and cakes with higher alteration also show higher elasticity (Baixauli et al., 2008).

Throughout storage the values for the colour parameter L* of the control muffins (no coating) and coated with formulations FS2 differed significantly ($p<0.05$). Whereas the muffins coated with formulations FS1 did not show significant difference ($p>0.05$, Table 6). The similarity in colour on formulations FS1 and FS2 are confirmed by the chromaticity a*, which indicated low values for the samples on crust and near the zero on the crumb. According to Baixauli et al., (2008) the higher the concentration of resistant starch in the dough, the lower the parameter a*.

On the analysis of the chromaticity parameter b*, it was noted that, regarding the crumb characteristics, the formulations FS1 and FS2 did not differ statistically between themselves ($p>0.05$). That way, it was noted that during storage for up to 48 days, there was significant difference ($p<0.05$) for the parameters L*, a* and b*, both for crust and for the crumb. This was expected, since the incorporation of the natural additives on the edible coating altered the colour of the muffins, due to the migration of the pigments derived from the soluble coffee and cocoa powder, which could have

Table 7. Shelf life of the control (no coating) and the muffins coated with the selected formulations (FS1 and FS2) in accelerated conditions (35°C) and in normal storage temperature (25°C).

Formulation	Shelf life (days)		
	Accelerated conditions (35°C)	Q_{10}	Normal conditions (25°C)
C	8	1.0	8
FS_1	24	2.18	33
FS_2	40	2.04	87

altered the volatile compounds, and therefore justified the preference of the tasters on the sensorial analysis (Table 2).

Shelf life of the muffins with edible active coatings

Controlled delivery of active agents into the food via packaging films for extended periods of storage and distribution restricts the development of undesirable flavours produced as a result of directly incorporating additives into the food (Majid et al., 2018). In addition, the use of edible films and coatings can enhance the process of preservation of food as shown in this work.

The counting of values for the mould and yeast and Wa of the control (no coating) and that of the muffins with selected active coatings (FS1 and FS2), under accelerated conditions (35°C) and normal storage temperature (25°C) were monitored at each 8 days. The values of the shelf life and the Q10 of the muffins coated with formulations FS1 and FS2 in accelerated conditions (35°C) and in normal storage temperature (25°C) are shown in Table 7. The formulation showing the shorter shelf life (8 days) in accelerated conditions was the control (no coating), which did not have any type of coating, also showing a shelf life of 8 days under normal storage conditions (25°C).

The coating that kept the muffins for long (40 days) in accelerated conditions was FS2 (0.76% soluble coffee, 0.22% cocoa powder and 0.82% propolis extract), with a shelf life of approximately 87 days in normal conditions of storage (25°C). This represents an increase of approximately 990 and 160% on the shelf life of products in relation to the control and formulation FS1, respectively.

The commercial brands of muffins show a best before date around 90 to 120 days, using additives such as potassium sorbate and sorbic acid incorporated to the formulations dough, with a maximum limit of 1.000 $mg.kg^{-1}$ (Codex Alimentarius, 2015). The result of the shelf life of muffins coated with formulation FS2 corresponds to the shelf life of the commercial brands, however without the use of these additives on its dough. This shows that the use of natural additives incorporated to the coating, in the tested concentrations, can exert the same function, representing an alternative to decrease the ingestion of synthetic preservatives and protect the consumer health.

In a recent study, muffins were developed from flax seed germinated and not germinated (Kaur et al., 2018). The prepared muffins were packed in linear low density polyethylene (LLDPE) and stored under ambient conditions and refrigerated for 1 month to evaluate the shelf life of the best levels. Even using LLDPE, only the muffins stored for 15 days under ambient conditions and for 1 month under refrigerated conditions had consumption conditions, demonstrating the efficiency of the material developed in this study for this type of food product.

In other work, cassava starch (and soy protein concentrate) edible coatings containing 20% glycerol was used in extending the shelf life of toasted groundnut (Chinma et al., 2014). Chemical indices of oxidative rancidity and sensory parameters were evaluated. The use of 50:50 (cassava starch:soy protein concentrate) edible coatings on toasted groundnut extended the shelf life of toasted groundnuts for 14 days compared to uncoated toasted groundnuts which developed objectionable taste after second day of storage at ambient (27 ± 1°C) condition.

The results obtained indicate that the edible active coatings based on cassava starch, plasticised with inverted sugar and sucrose, containing the natural active additives soluble coffee, cocoa powder and propolis extract, can be a competitive alternative to reduce the synthetic additives on the composition and preservation of muffins. The concentrations of the natural additives tested for the edible coatings reduce the water activity and humidity, fundamental properties for the storage of bakery products.

Conclusions

The edible active coating containing 0.76% soluble coffee, 0.22% cocoa powder and 0.82% propolis, effectively controlled the development of mould and yeast on the muffins without synthetics additives for 40 days in accelerated conditions. That resulted in a shelf life of 87 days in normal conditions, due to the antimicrobial action associated to the maintenance of the other properties of stability of the product. The shelf life of the commercial muffins containing synthetic additives is 90 days. The natural additives in the concentration used in this study

can exert the same function, representing an alternative to decrease the ingestion of synthetic preservatives. However, there is a need for additional studies to evaluate the action of these coatings in other bakery products, where the storage conditions, together with the products characteristics and its possible interactions with the coatings can demonstrate the real efficacy of the active coating.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Abdou ES, Elkholy SS, Elsabee MZ, Mohamed E (2008). Improved antimicrobial activity of polypropylene and cotton nonwoven fabrics by surface treatment and modification with chitosan. Journal of Applied Polymer Science 108(4):2290-2296. doi: 10.1002/app.25937

Abreu AS, Oliveira M, de Sá A, Rodrigues RM, Cerqueira MA, Vicente AA, Machado AV (2015). Antimicrobial nanostructured starch based films for packaging. Carbohydrate Polymers 129:127-134. doi: 10.1016/J.CARBPOL.2015.04.021

American Association of Cereal Chemists (2000). Method AACC 74-09: Measurement of Bread Firmness by Universal Testing Machine. In: Approved Methods of Analysis, 10th edn. American Association of Cereal Chemists, Saint Paul, MN:

Antoniou J, Liu F, Majeed H, Zhong F (2015). Characterization of tara gum edible films incorporated with bulk chitosan and chitosan nanoparticles: A comparative study. Food Hydrocolloids 44:309-319. doi: 10.1016/j.foodhyd.2014.09.023

Aydogdu A, Kirtil E, Sumnu G, Oztop MH, Aydogdu Y (2018). Utilization of lentil flour as a biopolymer source for the development of edible films. Journal of Applied Polymer Science 135:46356. doi: 10.1002/app.46356

Baik O, Marcotte M, Castaigne F (2000). Cake baking in tunnel type multi-zone industrial ovens Part II. Evaluation of quality parameters. Food Research International 33:599-607. doi: 10.1016/S0963-9969(00)00096-X

Baixauli R, Salvador A, Fiszman SM (2008). Textural and colour changes during storage and sensory shelf life of muffins containing resistant starch. European Food Research and Technology 226:523-530. doi: 10.1007/s00217-007-0565-4

Bajpai VK, Kamle M, Shukla S, Mahato DK, Chandra P, Hwang SK, Kumar P, Huh YS, Han YK (2018). Prospects of using nanotechnology for food preservation, safety, and security. Journal of Food and Drug Analysis 26:1201-1214. doi: 10.1016/J.JFDA.2018.06.011

Ban Z, Feng J, Wei W, Yang X, Li J, Guan J, Li J (2015). Synergistic Effect of Sodium Chlorite and Edible Coating on Quality Maintenance of Minimally Processed Citrus grandis under Passive and Active MAP. Journal of Food Science 80:C1705-C1712. doi: 10.1111/1750-3841.12956

Barba C, Eguinoa A, Maté JI (2015). Preparation and characterization of β-cyclodextrin inclusion complexes as a tool of a controlled antimicrobial release in whey protein edible films. LWT - Food Science and Technology 64:1362-1369. doi: 10.1016/j.lwt.2015.07.060

Bonilla J, Sobral PJA (2016). Antioxidant and physicochemical properties of blended films based on gelatin-sodium caseinate activated with natural extracts. Journal of Applied Polymer Science 134:. doi: 10.1002/app.44467

Channaiah LH, Michael M, Acuff JC, Phebus RK, Thippareddi H, Olewnik M, Milliken G (2017). Validation of the baking process as a kill-step for controlling Salmonella in muffins. International Journal of Food Microbiology 250:1-6. doi: 10.1016/J.IJFOODMICRO.2017.03.007

Chinma CE, Ariahu CC, Abu JO (2014). Shelf Life Extension of Toasted Groundnuts through the Application of Cassava Starch and Soy Protein-Based Edible Coating. Nigerian Food Journal 32:133–138. doi: 10.1016/S0189-7241(15)30106-5

Chiumarelli M, Pereira LM, Ferrari CC, Sarantópoulos CIGL, Hubinger MD (2010). Cassava starch coating and citric acid to preserve quality parameters of fresh-cut "Tommy atkins" mango. Journal of Food Science 75:E297–E304. doi: 10.1111/j.1750-3841.2010.01636.x

Codex Alimentarius (2015). Norma General para los Aditivos Alimentarios: Codex STAN 192-1995. FAO

Costa SS, Druzian JI, Machado BAS, De Souza CO, Guimaraes AG (2014). Bi-functional biobased packing of the cassava starch, glycerol, licuri nanocellulose and red propolis. PLoS ONE 9(11):1-10 doi: 10.1371/journal.pone.0112554

Da Silva JBA, Nascimento T, Costa LAS, Pereira FV, Machado BA, Gomes GVP, Assis DJ, Druzian JI (2015). Effect of Source and Interaction with Nanocellulose Cassava Starch, Glycerol and the Properties of Films Bionanocomposites. Materials Today: Proceedings 2(1):200-207. doi: 10.1016/j.matpr.2015.04.022

Esteller MS, Lannes SCDS (2005). Parâmetros complementares para fixação de identidade e qualidade de produtos panificados. Ciênc Tecn Aliment 25(4):802-806. doi: 10.1590/S0101-20612005000400028

Fakhouri FM, Martelli SM, Caon T, Velasco JI, Mei LHI (2015). Edible films and coatings based on starch/gelatin: Film properties and effect of coatings on quality of refrigerated Red Crimson grapes. Postharvest Biology and Technology 109:57–64. doi: 10.1016/j.postharvbio.2015.05.015

Farris S, Unalan UI, Introzzi L, Fuentes-Alventosa JM, Cozzolino CA (2014). Pullulan-based films and coatings for food packaging: Present applications, emerging opportunities, and future challenges. Journal of Applied Polymer Science 131(13):1-12 doi: 10.1002/app.40539

Femi-Adepoju FG, Olufemi Adepoju A (2014). Antifungal Activities of Seed Oil of Neem (Azadirachta indica A. Juss.). Global Journal of Biology, Agriculture & Health Sciences 3(1):106–109

Galus S, Kadzińska J (2015). Food applications of emulsion-based edible films and coatings. Trends in Food Science and Technology 45(2):273–283. doi: 10.1016/j.tifs.2015.07.011

Ganiari S, Choulitoudi E, Oreopoulou V (2017). Edible and active films and coatings as carriers of natural antioxidants for lipid food. Trends in Food Science & Technology 68:70-82. doi: 10.1016/J.TIFS.2017.08.009

Gélinas P, Roy G, Guillet M (1999). Relative effects of ingredients on cake staling based on an accelerated shelf-life test. Journal of Food Science 64(5):937–940. doi: 10.1111/j.1365-2621.1999.tb15944.x

Gutiérrez TJ, Morales NJ, Pérez E, Tapia MS, Famá L (2015). Physico-chemical properties of edible films derived from native and phosphated cush-cush yam and cassava starches. Food Packaging and Shelf Life 3:1-8. doi: 10.1016/j.fpsl.2014.09.002

Gutiérrez TJ, Suniaga J, Monsalve A, García NL (2016). Influence of beet flour on the relationship surface-properties of edible and intelligent films made from native and modified plantain flour. Food Hydrocolloids 54(PartB):234-244. doi: 10.1016/j.foodhyd.2015.10.012

Gutkoski LC, Teixeira DMF, Durigon A, Ganzer AG, Bertolin TE, Colla LM (2009). Influência dos teores de aveia e de gordura nas características tecnológicas e funcionais de bolos. Ciência e Tecnologia de Alimentos 29(2):254-261. doi: 10.1590/S0101-20612009000200003

IAL-Instituto Adolfo Lutz (2008). Métodos Físico-Químicos para Análise de Alimentos -4ª Edição 1ª Edição Digital, 4th edn. Instituto Adolfo Lutz, São Paulo, SP.

Jan KN, Panesar PS, Singh S (2018). Optimization of antioxidant activity, textural and sensory characteristics of gluten-free cookies made from whole indian quinoa flour. LWT 93:573-582. doi: 10.1016/J.LWT.2018.04.013

Karmaus AL, Osborn R, Krishan M (2018). Scientific advances and challenges in safety evaluation of food packaging materials: Workshop proceedings. Regulatory Toxicology and Pharmacology 98:80-87. doi: 10.1016/J.YRTPH.2018.07.017

Kaur A, Kaur R, Bhise S (2018). Baking and sensory quality of germinated and ungerminated flaxseed muffins prepared from wheat flour and wheat atta. Journal of the Saudi Society of Agricultural Sciences. doi: 10.1016/J.JSSAS.2018.07.002

Kaur R, Kaur M (2018). Microstructural, physicochemical, antioxidant, textural and quality characteristics of wheat muffins as influenced by partial replacement with ground flaxseed. LWT 91:278-285. doi: 10.1016/J.LWT.2018.01.059

King AD, Hocking AD, Pitt JI (1979). Dichloran-rose bengal medium for enumeration and isolation of molds from foods. Applied and Environmental Microbiology 37(5):959-964

Liu J, Liu X, Man Y, Liu Y (2018). Reduction of acrylamide content in bread crust by starch coating. Journal of the Science of Food and Agriculture 98(1):336-345. doi: 10.1002/jsfa.8476

Lopes AS (2005). Surinam cherru and west Indian cherry: study of processing, stability and formulation of a mixed nectar. Ph.D. Thesis, Campinas State University – UNICAMP.

Majid I, Ahmad Nayik G, Mohammad Dar S, Nanda V (2018). Novel food packaging technologies: Innovations and future prospective. Journal of the Saudi Society of Agricultural Sciences 17(4):454-462. doi: 10.1016/J.JSSAS.2016.11.003

Martínez-Cervera S, Salvador A, Sanz T (2014). Comparison of different polyols as total sucrose replacers in muffins: Thermal, rheological, texture and acceptability properties. Food Hydrocolloids 35:1-8. doi: 10.1016/j.foodhyd.2013.07.016

Ollé Resa CP, Gerschenson LN, Jagus RJ (2016). Starch edible film supporting natamycin and nisin for improving microbiological stability of refrigerated argentinian Port Salut cheese. Food Control 59:737-742. doi: 10.1016/j.foodcont.2015.06.056

Oriani VB, Molina G, Chiumarelli M, Pastore GM, Hubinger MD (2014). Properties of cassava starch-based edible coating containing essential oils. Journal of Food Science 79:E189-E194. doi: 10.1111/1750-3841.12332

Osawa CC, Fontes CB, Miranda EHW, Chang YK, Steel CJ (2009). Physical and chemical evaluation of chocolate cake covered with gelatin, stearic acid, modified starch or “carnaúba” was edible icing. Ciência e Tecnologia de Alimentos 29(1):92-99. doi: 10.1590/S0101-20612009000100015

Osorio FA, Molina P, Matiacevich S, Enrione J, Skurtys O (2011). Characteristics of hydroxy propyl methyl cellulose (HPMC) based edible film developed for blueberry coatings. Procedia Food Science 1:287-293. doi: 10.1016/J.PROFOO.2011.09.045

Pagno CH, Klug TV, Costa TMH, Rios AO, Flôres SH (2016). Physical and antimicrobial properties of quinoa flour-based films incorporated with essential oil. Journal of Applied Polymer Science 133(16): doi: 10.1002/app.43311

Parra DF, Tadini CC, Ponce P, Lugão AB (2004). Mechanical properties and water vapor transmission in some blends of cassava starch edible films. Carbohydrate Polymers 58(4):475-481. doi: 10.1016/J.CARBPOL.2004.08.021

Ramírez-Guerra HE, Castillo-Yañez FJ, Montaño-Cota EA, Ruíz-Cruz, S, Márquez-Ríos E, Canizales-Rodríguez DF, Torres-Arreola W, Montoya-Camacho N, Ocaño-Higuera VM (2018). Protective Effect of an Edible Tomato Plant Extract/Chitosan Coating on the Quality and Shelf Life of Sierra Fish Fillets. Journal of Chemistry 2018. doi: 10.1155/2018/2436045

Rangel-Marrón M, Mani-López E, Palou E, López-Malo A (2019). Effects of alginate-glycerol-citric acid concentrations on selected physical, mechanical, and barrier properties of papaya puree-based edible films and coatings, as evaluated by response surface methodology. LWT 101:83-91. doi: 10.1016/J.LWT.2018.11.005

Razavi SMA, Mohammad Amini A, Zahedi Y (2015). Characterisation of a new biodegradable edible film based on sage seed gum: Influence of plasticiser type and concentration. Food Hydrocolloids 43:290-298. doi: 10.1016/j.foodhyd.2014.05.028

Resende NS, Gonçalves GAS, Reis KC, Tonoli GHD, Boas EVBV (2018). Chitosan/Cellulose Nanofibril Nanocomposite and Its Effect on Quality of Coated Strawberries. Journal of Food Quality 2018. doi: 10.1155/2018/1727426

Romanazzi G, Feliziani E, Santini M, Landi L (2013). Effectiveness of postharvest treatment with chitosan and other resistance inducers in the control of storage decay of strawberry. Postharvest Biology and Technology 75:24-27. doi: 10.1016/J.POSTHARVBIO.2012.07.007

Sapper M, Talens P, Chiralt A (2019). Improving Functional Properties of Cassava Starch-Based Films by Incorporating Xanthan, Gellan, or Pullulan Gums. 2019:1-9 https://doi.org/10.1155/2019/5367164

Sluimer P(2005). Principles of Breadmaking: Functionality of Raw Materials And Process Steps. St Paul: AACC Inc. 212 p. http://extbooktor.com/principles-of-breadmaking-functionality-of-raw-materials-and-process-steps-piet-sluimer-libraries-began-providing-books.pdf

Sorour H, Tanaka F, Uchino T (2014). Impact of Non-Thermal Processing on the Microbial and Bioactive Content of Foods. Global Journal of Biology, Agriculture & Health Sciences 3(1):153-167

Sun Q, Xi T, Li Y, Xiong L (2014). Characterization of corn starch films reinforced with CaCO3 nanoparticles. PLoS ONE 9:e106727. doi: 10.1371/journal.pone.0106727

Suseno N, Savitri E, Sapei L, Padmawijaya KS (2014). Improving Shelf-life of Cavendish Banana Using Chitosan Edible Coating. Procedia Chemistry 9:113–120. doi: 10.1016/J.PROCHE.2014.05.014

Tian S, Chen Y, Chen Z, Yang Y, Wang Y (2018). Preparation and Characteristics of Starch Esters and Its Effects on Dough Physicochemical Properties. 2018: https://doi.org/10.1155/2018/1395978

Treviño-Garza MZ, García S, Flores-González M del S, Arévalo-Niño K (2015). Edible Active Coatings Based on Pectin, Pullulan, and Chitosan Increase Quality and Shelf Life of Strawberries (Fragaria ananassa). Journal of Food Science 80(8):M1823-30. doi: 10.1111/1750-3841.12938

Valenzuela C, Tapia C, López L, Bunger A, Escalona V, Abugoch L (2015). Effect of edible quinoa protein-chitosan based films on refrigerated strawberry (Fragaria × ananassa) quality. Electronic Journal of Biotechnology 18(6):406-411. doi: 10.1016/J.EJBT.2015.09.001

Valerini D, Tammaro, L, Di Benedetto F, Vigliotta G, Capodieci L, Terzi, R, Rizzo A (2018). Aluminum-doped zinc oxide coatings on polylactic acid films for antimicrobial food packaging. Thin Solid Films 645:187-192. doi: 10.1016/J.TSF.2017.10.038

Veiga-Santos P, Oliveira LM, Cereda MP, Scamparini ARP (2007). Sucrose and inverted sugar as plasticizer. Effect on cassava starch–gelatin film mechanical properties, hydrophilicity and water activity. Food Chemistry 103(2):255-262. doi: 10.1016/j.foodchem.2006.07.048

Veiga-Santos P, Suzuki CK, Cereda MP, Scamparini ARP (2005). Microstructure and color of starch–gum films: Effect of gum deacetylation and additives. Part 2. Food Hydrocolloids 19(6):1064-1073. doi: 10.1016/J.FOODHYD.2005.02.00

Vilela C, Kurek M, Hayouka Z, Röcker B, Yildirim S, Antunes MDC, Nilsen-Nygaard J, Pettersen MK, Freire CSR (2018). A concise guide to active agents for active food packaging. Trends in Food Science & Technology 80:212-222. doi: 10.1016/j.tifs.2018.08.006

Vital ACP, Guerrero A, Kempinski EMBC, Monteschio JO, Sary C, Ramos TR, Campo MDM, Prado IND (2018). Consumer profile and acceptability of cooked beef steaks with edible and active coating containing oregano and rosemary essential oils. Meat Science 143:153-158. doi: 10.1016/J.MEATSCI.2018.04.035.

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Genetic diversity of antibiotic and plant extract resistant *Staphylococcus aureus* isolated from hospitalized patients in Ekiti State, Nigeria

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This study aimed to determine the genetic diversity of antibiotics and plants extract resistant *Staphylococcus aureus* using molecular technique. A total of 106 human clinical samples were collected from patients in three different hospitals within Ekiti State. Microbiological and molecular analyses were performed using standard methods. Antimicrobial susceptibility test with common antibiotics and plant leaves extracts was carried out using disk and agar well diffusion methods. Urine samples from male patients recorded high percentage of *S. aureus* (57.1%) as compared to the female patients (43.1%). Percentage of *S. aureus* recorded from the ear swab samples in male patients (58.3%) was also high as compared to the female (41.7%). From the nose swab samples, female patients recorded 60% as compared to their male counterpart (40%). Equal percentage (50%) of wound infection caused by *S. aureus* was observed in both male and female patients. Of the eight antibiotics used in this study, the *S. aureus* isolated were susceptible to ofloxacin (25 to 36 mm), gentamycin (16 to 23 mm) and erythromycin (11 to 25 mm). All the isolates confirmed resistance to ceftaxidime and cloxacillin. Nine isolates were susceptible to cefuroxime with zones of inhibition that ranged from 10 to 25 mm, while 11 were susceptible to ceftriaxone with zone of inhibition between 11 and 20 mm. Only one isolate was sensitive to augmentin (amoxicillin/clavulanate) with zone of inhibition of 20 mm. Out of three plant extracts used in this study, aqueous leaf extract of *Terminalia catappa* demonstrated highest antibacterial activity on the test isolates with zone of inhibition of 16 to 36 mm followed by *Mangifera indica* with inhibition ranging from 11 to 32 mm, while least inhibition ranging from 11 to 20 mm was exhibited by *Acalypha wikesiena*. Random amplified polymorphic DNA (RAPD) proved to be useful as genetic markers in determining genetic diversity among antibiotic and plant extract resistant *S. aureus*.

Key words: *Acalypha wikesiena,* multidrug-resistant strains, *Staphylococcus aureus,* genetic diversity.

INTRODUCTION

Modern medicine is no doubt faced with an unprecedented threat posed by multidrug-resistant bacteria (Levy and Marshall, 2004). It has been advised that a multidisciplinary approach is required to tackle the

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emergence and evolution of drug resistance microorganisms (Martinez and Baquero, 2002). In their study, Baquero et al. (2003) averred that the multidisciplinary approach will be a challenge given that the trend of antibiotic resistance appears as a reflection of the characteristic of a dynamic, highly complex, and self-organizing system evolving at an instance of complex situations. New molecular technologies promise to offer adequate and effective tools in the fight against antibiotic resistance strains of microorganisms. Advance molecular approaches have also been reported to be effective containment of multidrug-resistant strains, given the accurate detection of bacterial species from different environment. Currently, the main advantage of molecular resistance testing is the rapid turn-around time and improved accuracy, both of which are relevant particularly in cases of life-threatening diseases such as meningitis and sepsis, for which rapid detection, identification and resistance testing is important (Johansen et al., 2003). It is noteworthy to add that, rapid polymerase chain reaction (PCR)-based assays for resistance testing have been used as excellent complementary tools in the laboratory for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) (Warren et al., 2004).

Medicinal plants as alternative therapeutic agent have recently attracted the attention of the biological scientific communities. Use of medicinal plants involves the isolation and identification of secondary metabolites produced by plants and is used as active principles in medicinal preparations. Application of various herbal medicinal preparations for the treatment of *S. aureus* associated illnesses in Nigeria has been reported (Akinyemi et al., 2005). Volumes of reports have demonstrated the antimicrobial properties of several of these plants against multi-drug resistant bacteria, particularly methicillin resistant *S. aureus* (Ankri and Mirelman, 1999; Adesino et al., 2011; Khan et al., 2016).

Methicillin resistant *S. aureus* (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans. MRSA is any strain of *S. aureus* that has developed through the horizontal gene transfer and natural selection, multi-resistance to beta-lactam antibiotics, which include the penicillins (methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalosporins. Understanding the *S. aureus* lineages therefore, is crucial in controlling nosocomial infections. Recent studies on *S. aureus* in Nigeria have revealed an escalating burden of MRSA. However, the *S. aureus* genotypes circulating among hospitalized patients in hospitals found in Ekiti State are not known, hence this study.

MATERIALS AND METHODS

Sampling

In this research, a total of 106 human clinical samples were collected and transported within 2 h of collection to the Microbiology Research Laboratory in the Department of Biological Sciences, Afe Babalola University, Ado-Ekiti for microbiological analysis. The samples used in this research were urine, wound, nose and ear swabs obtained from patients hospitalized at different wards of Ekiti State University Teaching Hospital (EKSUTH), State Specialist Hospital Ikere-Ekiti, and General Hospital Iyin-Ekiti in Ekiti State Nigeria. Samples were obtained from both male and female of different ages. From men, 33 urine samples, 14 ear swabs, four nose swabs and seven wound swabs were obtained. On the other hand, a total of 45 samples were obtained from women comprising 25 urine samples, ten ear swabs, six nose swabs and seven wound swabs. Each urine sample was aseptically collected into a sterile sampling bottle, while ear, nose and wound swabs were collected using a sterile swab stick.

Plant sample collection and preparation

Fresh plant leaves of *Terminalia catappa* (Almond), *Mangifera indica* (Mango) and *Acalypha wikesiena* (Acalypha) were obtained within the premises of Afe Babalola University and identified in the Department of Botany, University of Ibadan, Nigeria. Voucher specimens for *T. catappa* (UIH 22567), *M. indica* (UIH 22568) and *A. wikesiena* (UIH 22569) were deposited at University of Ibadan Herbarium of the Department of Botany. The leaves were brought to the laboratory, stripped from their stems, washed with distilled water and crushed into a fine infusion using a laboratory mortar and pestle. The solution was filtered with No. 1 Whatman filter paper and finally passed through membrane filter to obtain sterile extract. These were labelled and kept at 4°C in the refrigerator for use. A clean filter was used to obtain the extracts respectively into three well labelled beakers.

Qualitative analysis of phytochemical constituent

Simple standard chemical tests as previously described by Harborne (1973) and Chethana et al. (2013) were employed for detecting the presence of some phytochemical components such as saponins, tannins, alkaloids, steroids, phenol, terpenoid, flavonoid and glycosides in the plant extract.

Isolation and identification of test organisms

The isolates gotten from the various sites were identified using standard microbiological method as described by Cheesbrough (2000).

Antibiotic sensitivity testing

Antimicrobial susceptibility of the isolates was determined by the Kirby Bauer disc diffusion method according to CLSI guidelines (2012) using the following antibiotics: ofloxacin (OFL), augmentin (AUG), ceftaxidime (CAZ), cefuroxime (CRX), gentamycin (GENT), ceftriaxone (CTR), erythromycin (ERY) and cloxacillin (CXC). Young culture of the isolate was suspended in a sterile saline solution (0.85% NaCl). The suspension was adjusted to match 0.5 McFarland turbidity standards. The antibiotics discs were aseptically placed on Mueller Hinton agar surface using sterile forceps and incubate at 37°C for 24 to 48 h. Effectiveness of the antibiotics was shown by clear zones around them which were measured and interpreted as either resistance, intermediate, or susceptible.

Plant extract inhibitory test

Agar well diffusion method was used as previously described

(Magaldi et al., 2004; Valgas et al., 2007) to evaluate the antimicrobial activity of the plants extracts. Clear zones of inhibition after incubation were measured as previously described (Lino and Deogracious, 2006).

DNA isolation and purification of bacterial genomic DNA

Sterile dry swabs were used for streaking clinical samples onto sterile nutrient agar plates (CONDA). Inoculated plates were incubated at 37°C for 48 h. Stock cultures of the pure isolates were prepared and refrigerated at 4°C to be kept for further analysis. Extraction and purification of genomic DNA of the bacterial isolates was carried out using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo). The procedure employed was as described by the manufacturer.

RAPD PCR analysis

RAPD PCR analysis used in this study was performed as previously described by Onasanya et al. (2003). DNA primers tested were purchased from Operon Technologies (Alemada, Califonia, USA) with each being 10 nucleotides long. Two separate concentrations of DNA, 24 and 96 ng per reaction were used to test reproducibility as well as eliminate irregular amplification products from the analysis. Thirteen primers were screened for their ability to amplify the DNA of the *S. aureus* isolate. Six out of the 13 screened primers were found useful because they showed polymorphism. These were used for amplification of DNA of the 20 isolates. Amplification were performed in 25-µl reaction mixture consisting of genomic DNA, IX reaction buffer, (Promega), 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM Operon random primer, 2.5 µM $MgCl_2$ and 0.2 µl (1 unit) of Taq polymerase (Boehringer, Germany). A single primer was used in each reaction. Amplification was performed in a thermowell microtiter plate (Costa Corporation) using a Perkin Elmer programmable Thermal Controller model 9600. The cycling program was set as follows: initial denaturation at 94°C for 3 min followed by 45 cycles each at 94°C for 1 min, 40°C for 1 min., 72°C for 2 min and final extension at 72°C for 7 min. Amplified DNA products were analyzed by electrophoresis in 1.4% agarose gel using TAE buffer (45 Mm Tris-acetate, 1 Mm EDTA, pH 8.0) at 100 V. Gels were visualized by staining with ethidium bromide solution (0.5 µg/ml) and banding patterns were observed using the Photogel documentation system.

Phylogenetic analysis

Positions of unequivocally scorable RAPD bands were transformed into a binary character matrix (to indicate the presence or absence of a band at a particular position). Pair-wise distance matrices were compiled by the Numerical Taxonomy System (NTSYS) 2.0 software (Rohfl, 2000) using a simple matching coefficient for estimation by means of the Jaccard's coefficient to construct a similarity matrix (Ivchenko and Honov, 1998). Cluster analysis and dendrogram were produced on the basis of the unweighted pair group method arithmetic (UPGMA) (Sneath and Sokal, 1973; Jako et al., 2009) as the banding profiles of the six RAPD primers represent a novel approach for rapid screening of the multidrug resistant.

Statistical analysis

Data generated were expressed in mean ± standard error mean (SEM). One-way analysis of variance (ANOVA) followed by Student T-test (Walson, 1989; AOAC 1990) was used for this research.

RESULTS AND DISCUSSION

All samples analyzed for the presence of *Staphylococcus* on mannitol salt agar and nutrient agar media showed obvious bacterial growths. Out of 106 collected samples from both male and female patients of different age brackets admitted to the three hospitals within Ekiti State, Ekiti State University Teaching Hospital (EKSUTH) had the highest number of patient colonized with *S. aureus.*

From urine samples, high percentage of *S. aureus* in male patients (57.1%) as compared to their female counterpart (43.1%) was recorded. Percentage of *S. aureus* recorded from the ear swab samples in male patients (58.3%) was also high as compared to the female (41.7%). From the nose swab samples, female patients recorded 60% as compared to their male counterpart (40%). Equal percentage (50%) of wound infection caused by *S. aureus* was observed in both male and female patients

Further to this study, 20 representatives of the *S. aureus* isolates were picked for multidrug resistance pattern to antibiotics and plant leaves aqueous extracts of which 9 were obtained from patients' urine, three from the noses, four from the ears and four from wounds. These isolates were code-numbered accordingly. All the isolates were completely resistant to CAZ and CXC as there was no zone of inhibition observed. Nine isolates were observed to be sensitive to CRX with zones of inhibition ranging from 10 to 25 mm, while 11 isolates were sensitive to CTR with range between 11 and 20 mm. Meanwhile, only one isolate was sensitive to AUG 20 mm zone of inhibition (Figure 1). However, the 20 *S. aureus* isolates used in the study were all susceptible to OFL with clear zones of inhibition ranging from 24 to 35 mm followed by GEN 16 to 23 mm and ERY 11 to 25 mm.

This apparently high level of susceptibility of the isolates to OFL suggest that ofloxacin (OFL) could be a drug of choice for treating infections caused by *S. aureus* in the study area, especially at the present time, when *S. aureus* strains are resistant to other commonly used antibiotics as reported in many literatures. This finding is consistent with previous reports of Amadi et al. (2007) and Chigbu and Ezeronye (2003). Also, 100% sensitivity of *S. aureus* isolates to ofloxacin (OFL) was reported by Obi et al. (1996). In addition, high inhibition of OFL against *S. aureus* isolates was also highlighted by Chalita et al. (2004). However, low sensitivity was reported among most strains of methicillin resistant *S. aureus* (MRSA) isolated from patients with ocular infections (Uwaezuoke and Aririatu, 2004). *S. aureus* susceptibility to gentamycin (GEN) in this present study compares favourably with reports published in the South-western part of Nigeria, by Paul et al. (1982) and Ndip et al. (1997).

On the other hand, the sensitivity pattern of 20 *S. aureus* isolates to leaves extract shows that *T. catappa*

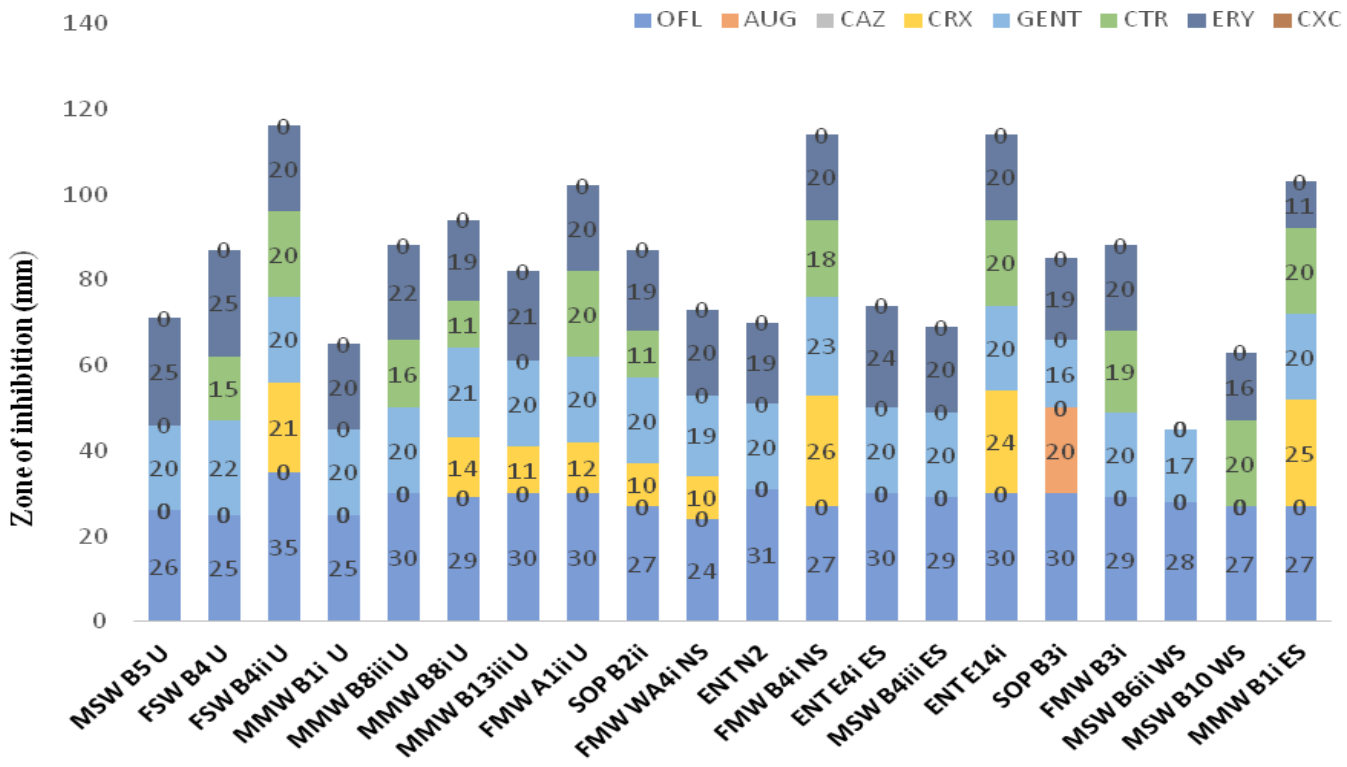

Figure 1. Susceptibility pattern of *S. aureus* to antibiotics. MSW = Male surgical ward; FSW = female surgical ward; MMW = male medical ward; FMW= female medical ward; SOP= surgical outpatient; ENT = ear nose and throat, B, A, W (5, 4, 1, 8, 3, 6, 10 respectively iBed no.; ES= ear swab; NS=nose swab; WS= wound swab. OFL = Ofloxacin; AUG = augmentin; CAZ = ceftaxidime; CRX = cefuroxime; GENT = gentamycin; CTR = ceftriaxone; ERY = erythromycin; CXC = cloxacillin.

demonstrated the highest level of effectiveness against 18 isolates which included isolates gotten from urine, ear, nose and wounds with zone of inhibition that ranges between 16 to 36 mm, with 2 isolates resistant to the plant. Although, 19 isolates were inhibited by *M. indica,* their zone of inhibition ranged between 11 and 32 mm, with only one isolate resistant and 14 out of the 20 isolates showed resistance to the aqueous leaf extract of *A. wikesiena*. Susceptibility of 6 isolates to *A. wikesiena* ranged from 11 to 20 mm zones of inhibition (Figure 2). The highest antimicrobial activity was observed with *T. catappa* which had better effect on 18 isolates with wider zones of inhibition than all the commercial antibiotics used in this study. Also, aqueous leaf extract of *M. indica* demonstrated higher antimicrobial effect on 19 isolates and its inhibitory activity was better than all the antibiotics used except OFL from which a wider zone of inhibition was observed. This indicates that the extracts of the leaf are as effective as the commercially prepared antibiotics thus confirming its potency in the treatment of *S. aureus*-mediated infections as well as gastroenteritis and skin dermatitis. Moreover, since the leaf extracts exhibited inhibitory activity on the test organisms, the antibacterial activity appears to be broad spectrum (Oluduro et al., 2011). The phytochemicals detected in the leaves aqueous extracts of *T. catppa, M. indica* and *A. wikesiena* in this study corresponds to earlier studies carried out with other plants by Onah et al. (2002), Akpan and Usoh (2004) and Oyewole et al. (2008).

The quantity of tannin present in *T. catappa* was significantly different from the quantity of tannin present in *A. wikesiena* but not significantly different from the value obtained in *M. indica* (Table 1). Furthermore, flavonoids content in *T. catappa* shows no significant difference from that in *M. indica*. No significant difference existed in the phenol quantity between *T. catappa, M. indica* and *A. wikesiena*. Also, the quantity of saponin in *T. catappa* was not significantly different from the values of *M. indica* and *A. wikesiena* (Table 1).

In this study, it was observed that all isolates from patients' samples receiving treatment from EKSUTH expressed similar pattern in their resistance to the antibiotic and plant extract used. Genetic fingerprinting of the 20 *S. aureus* isolates from urine, ear, nose and wound swabs of patients receiving treatment from 3 hospitals within Ekiti State was compared using RAPD-PCR markers. Of the 13 arbitrary primers tested, six primers (Table 2) showed a good level of polymorphism and reproducibility. The amplification reaction of 6 oligonucleotide primers yielded 135 bands of which 121 were polymorphic with sizes ranging between 200 and 3,000 bp (Figure 3a to f). 121 polymorphic RAPD

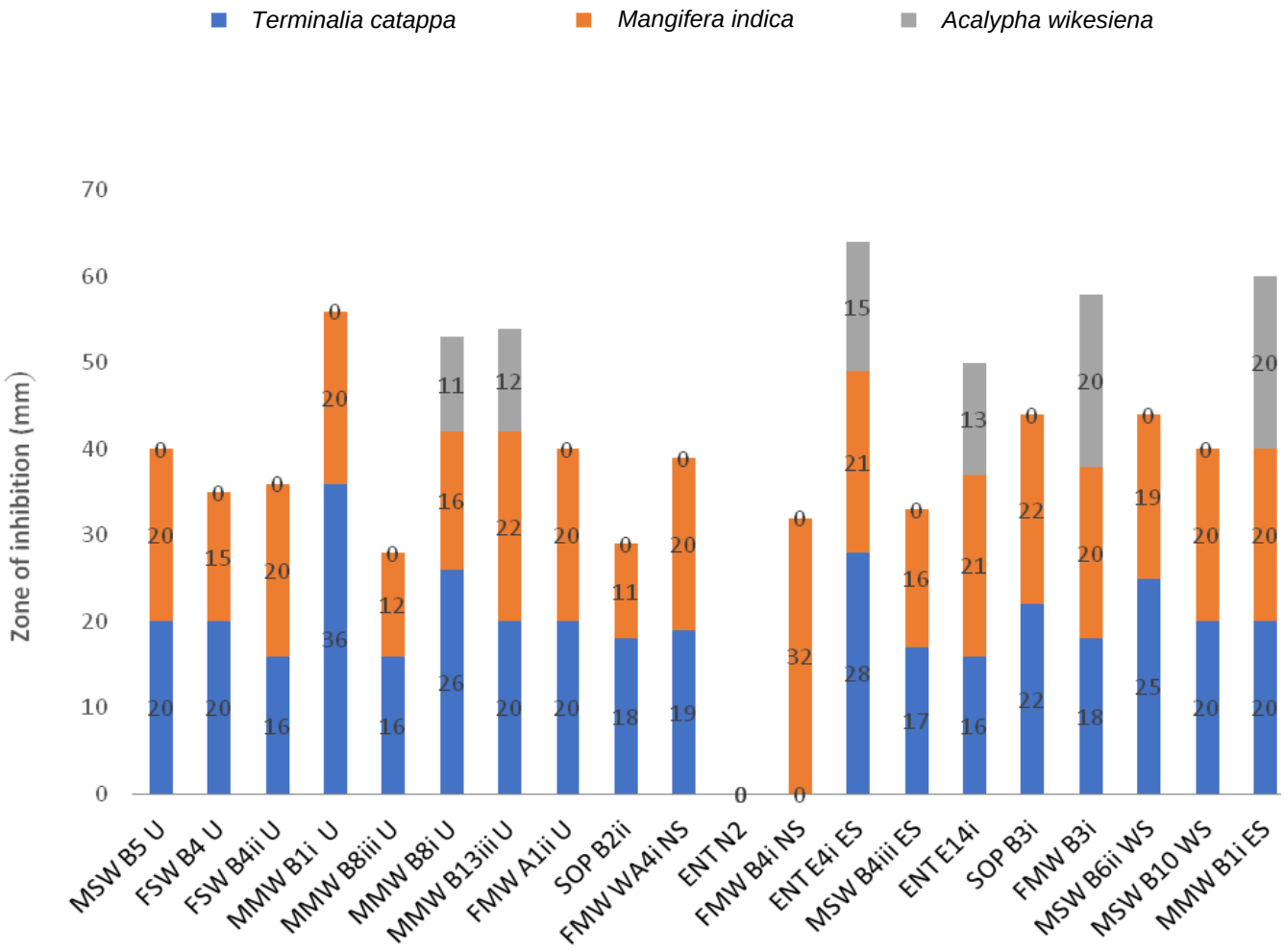

Figure 2. Antibacterial effect of *T. catappa, M. inidica* and *A. wikesienia* leaves aqueous extracts on the twenty *S. aureus* isolates. MSW= male surgical ward; FSW= female surgical ward; MMW=male medical ward; FMW= female medical ward; SOP= surgical out patient; ENT= ear nose and throat, B, A, W (5, 4,1, 8, 3, 6 and 10 respectively) = Bed no.; ES= ear swab; NS=nose swab; WS= wound swab.

Table 1. Quantity of phytochemicals from the aqueous leaf extracts (mg/g).

Samples	Tannin	Flavonoids	Phenol	Saponin	Alkaloids
T. catappa	0.04 ±0.01[a]	0.17 ±0.02[a]	0.03± 0.01[a]	2.60 ± 0.02[b]	3.63± 0.02[a]
M. indica	0.03±0.01[a]	0.17± 0.01[a]	0.03 ± 0.01[a]	3.31 ± 0.36[a]	2.43±0.40[b]
A. wikesiena	0.06± 0.01[b]	-	0.04 ± 0.02[a]	3.26 ± 0.01[a]	3.52±0.01[a]
S. V. (α = 0.05)	(p = 0.002)	0.000	0.868	0.096	0.089

Values are mean ± standard deviations of triplicate determinations. Values with different superscript per column are statistically significant (p<0.05).

markers were used to construct the phylogenetic relationship dendrogram among 20 *S. aureus* isolates into different group's genotypes and sub groups depending on their reaction with antibiotics and plants extract used in the study.

From the reaction of 8 antibiotics on the 20 *S. aureus* isolates, two different reactive groups emerged which consists of antibiotics reaction group 1 (ARg1) and antibiotics reaction group 2 (ARg2) at 75% similarity (Figure 4a). ARg1 yielded 3 subgroups which include ARg1a, ARg1b and ARg1c. ARg1a consisted of six isolates (1-MSWB5U, 4-MMWB1iU, 11-ENTN2, 13-

Table 2. Oligonucleotide primers showing levels of genetic diversity among *S. aureus* isolates using the RAPD-PCR analysis.

Operon primer code	Primer sequence 5' – 3'	Total number of fragments	Monomorphic bands	Polymorphic bands
OPT06	CAAGGGCAGA	25	3	22
OPT14	AATGCCGCAG	28	2	26
OPT01	GGGCCACTCA	22	2	20
OPB04	GGACTGGAGT	20	4	16
OPH08	GAAACACCCC	22	1	21
OPT12	GGGTGTGTAG	18	2	16
	Total	135	14	121

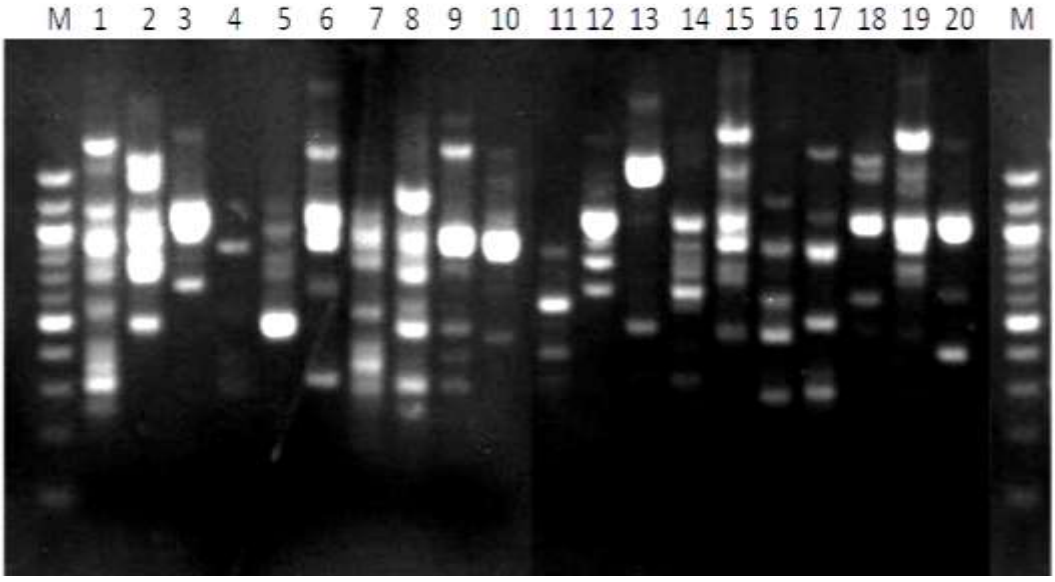

Figure 3a. Band patterns obtained after RAPD amplification of the 20 *S. aureus* isolates with the oligonucleotide OPT06. Lane M, DNA molecular size marker 1 kbp; Lane 1 – 20, 20 *S. aureus*

ENTE4iES, 17-FMWB3i and 14-MSWB4iiiES). The reactions of these isolates with five antibiotics were identical. ARg1a consisted of four isolates (2-FSWB4U, 5-MMWB8iiiU, 18-MSWB6iiWS and 19-MSWB10WS) that were also identical in their reaction with 5 antibiotics. ARg1c had only 1 isolate (16-SOPB3i). ARg2 yielded only 2 subgroups consisting of ARg2a and ARg2b. ARg2a consisted of 7 isolates (3-FSWB4iiU, 6-MMWB8iU, 20-MMWB1iES, 15-ENTE14i, 12-FMWB4iNS, 9-SOPB2ii and 8-FMWA1iiU). These isolates show similar pattern of activities in their reaction with the 5 antibiotics. ARg1a consisted of two isolates (7-MMWB13iiiU and 10-FMWWA4iNS). Both isolates in ARg2b were identical in their reaction with the five antibiotics.

Figure 4b presents cluster E that shows the reaction of 3 plant leaf extracts (*T. cattapa, M. indica* and *A. wikesiena*) on 20 *S. aureus* isolates. At 55% coefficient of similarity, 2 major groups were formed, namely ERg1 and ERg2. ERg1 developed two subgroups namely ERg1a and ERg1b. ERg1a which consists of 12 isolates (1-MSWB5U, 2-FSWB4U, 3-FSWB4iiU, 4-MMWB1iU, 17-FMWB3i, 18-MSWB6iiWS, 14-MSWB4iiiES, 16-SOPB3i, 10-FMWWA4iNS, 9-SOPB2ii, 8-FMWA1iiU and 5-MMWB8iiiU). These isolates reacted in the same way to the aqueous leaf extracts. ERg1b consisted of six isolates (6-MMWB8iU, 7-MMWB13iiiU, 20-MMWB1iES, 19-MSWB10WS, 13-ENTE4iES and 15-ENTE14i) which also reacted in the same way with the extracts. ERg2 had two isolates (11-ENT N2 and 12-FMWB4iNS) which reacted differently to the extracts.

Figure 4c presents cluster G which consists of two major group genotypes (Gg) represented as Gg1 and Gg2 at 10.5% similarity. Gg1 yielded 2 subgroups namely Gg1a and Gg1b at 11% similarity. Gg1a further gave rise to two subgroups consisting of Gg1ai and

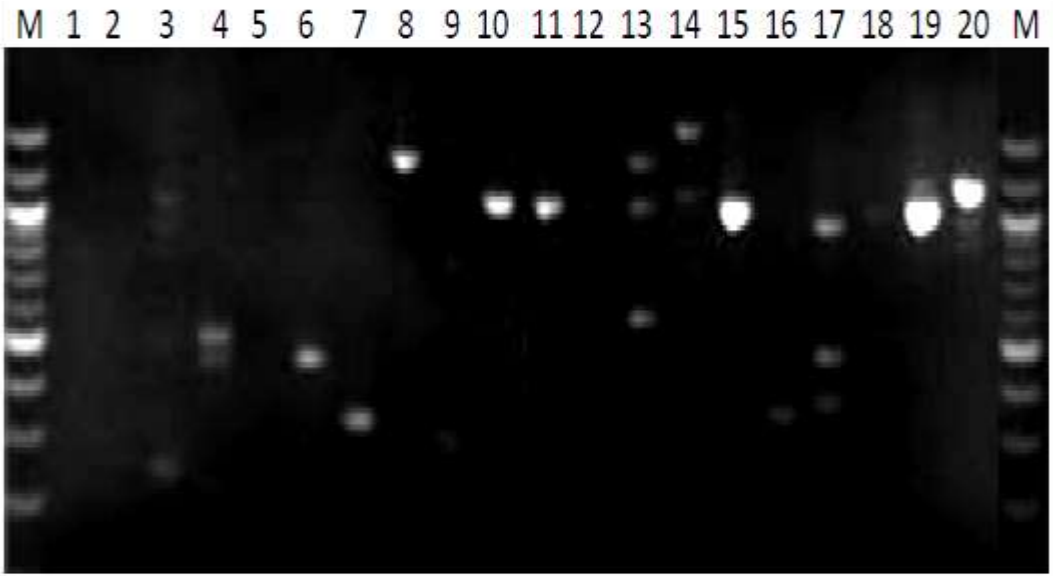

Figure 3b. Band patterns obtained after RAPD amplification of the 20 *S. aureus* isolates with the oligonucleotide OPH08. Lane M, DNA molecular size marker 1 kbp; Lane 1 – 20, 20 *S. aureus.*

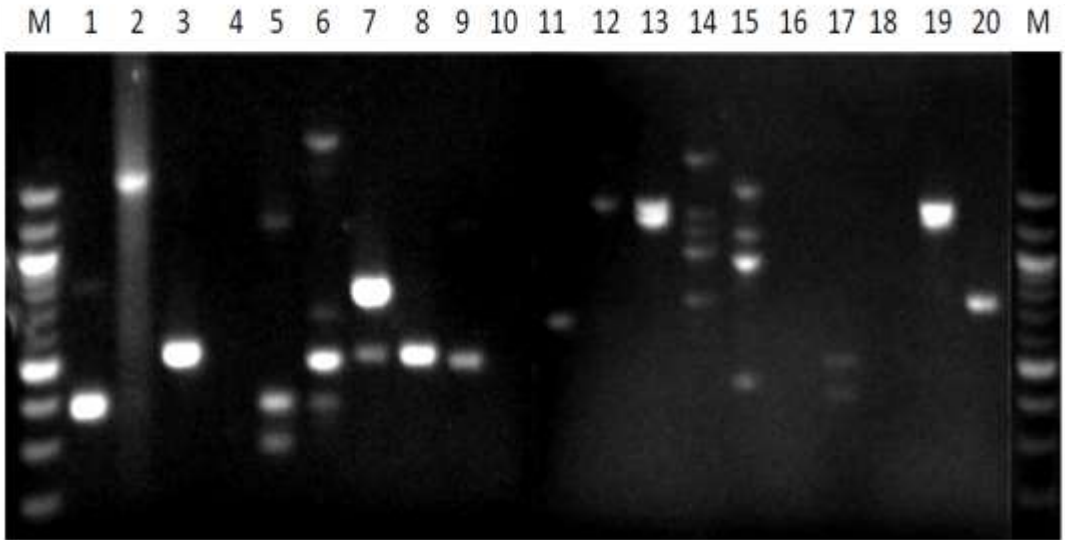

Figure 3c. Band patterns obtained after RAPD amplification of the 20 *S. aureus* isolates with the oligonucleotide OPB04. Lane M, DNA molecular size marker 1 kbp; Lane 1 – 20, 20 *S. aureus.*

Gg1aii; Gg1b gave rise also to two subgroups Gg1bi and Gg1bii as with 11.5% similarity. Gg2 did not yield any obvious subgroups. However, the (Gg) cluster formed three distinct groups; the first group consisted of four isolates (1, 16, 15 and 19), the second group consisted of four isolates (2, 6, 7 and 3) while the third group consisted of 9 isolates (8, 9, 17, 10, 11, 12, 18, 13 and 14). From the first group, it was observed that only the group genotype of isolate 1 was resistant to the antibiotics, making the group an antibiotic resistance emergent group. Group genotypes of the isolates in group 2 revealed antibiotics susceptibility because no resistance was found in the isolates.

Molecular research studies indicate that resistance is a complex system, resulting from multiple interacting mechanisms at the genomic, regulatory and expression levels. If the complementary effort of both the culture-dependent and molecular technique as evidenced in this study is anything to go by, molecular assays for resistance testing will not replace conventional culture-based antibiotic susceptibility testing in the immediate future (Fluit et al., 2001).

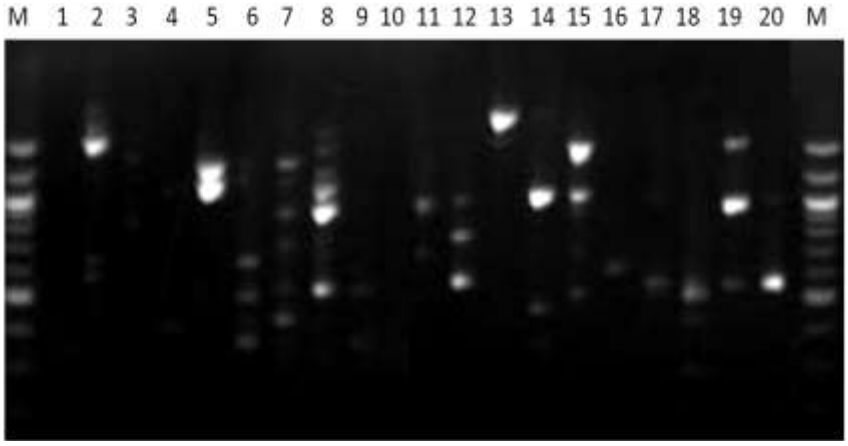

Figure 3d. Band patterns obtained after RAPD amplification of the 20 *S. aureus* isolates with the oligonucleotide OPT01. Lane M, DNA molecular size marker 1 kbp; Lane 1 – 20, 20 *S. aureus.*

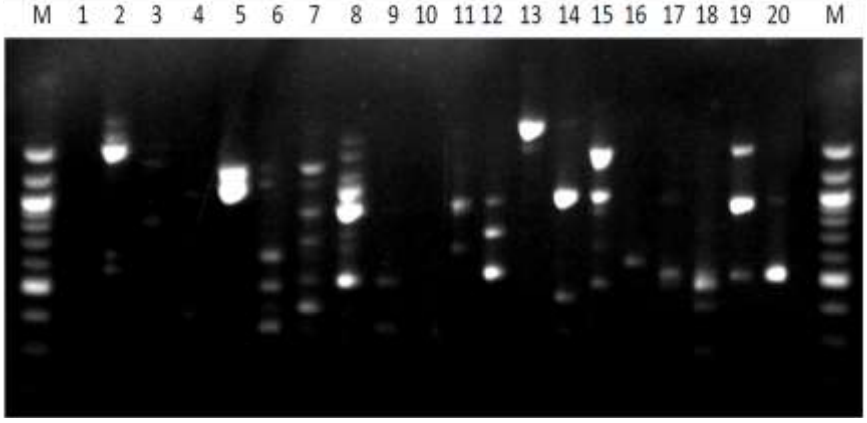

Figure 3e. Band patterns obtained after RAPD amplification of the 20 *S. aureus* isolates with the oligonucleotide OPT12, Lane M, DNA molecular size marker 1 kbp, Lane 1 – 20, 20 *S. aureus.*

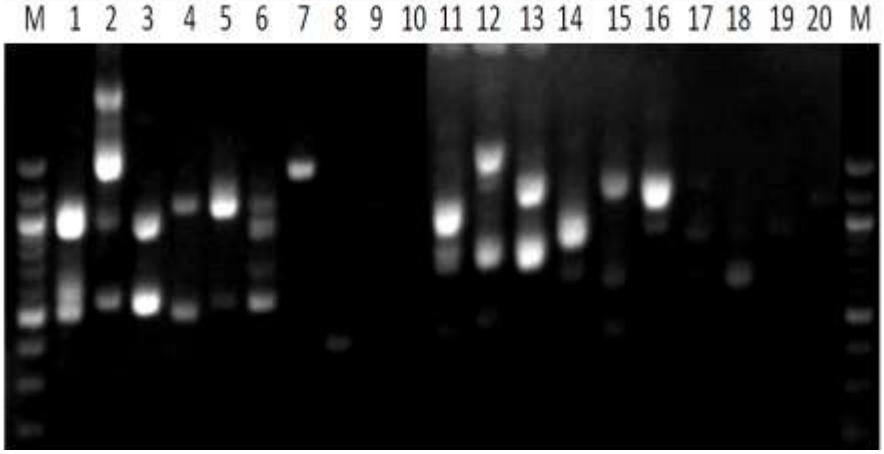

Figure 3f. Band patterns obtained after RAPD amplification of the 20 *S. aureus* isolates with the oligonucleotide OPT14. Lane M, DNA molecular size marker 1 kbp, Lane 1 – 20, 20 *S. aureus.*

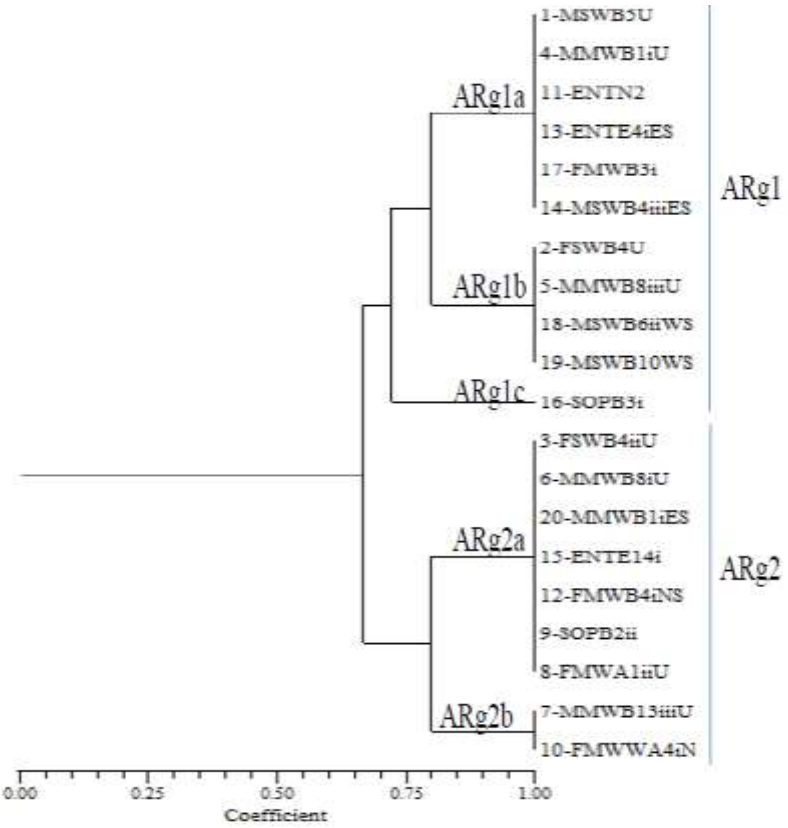

Figure 4a. Cluster A showing the reaction of twenty *S. aureus* isolates against five antibiotics.

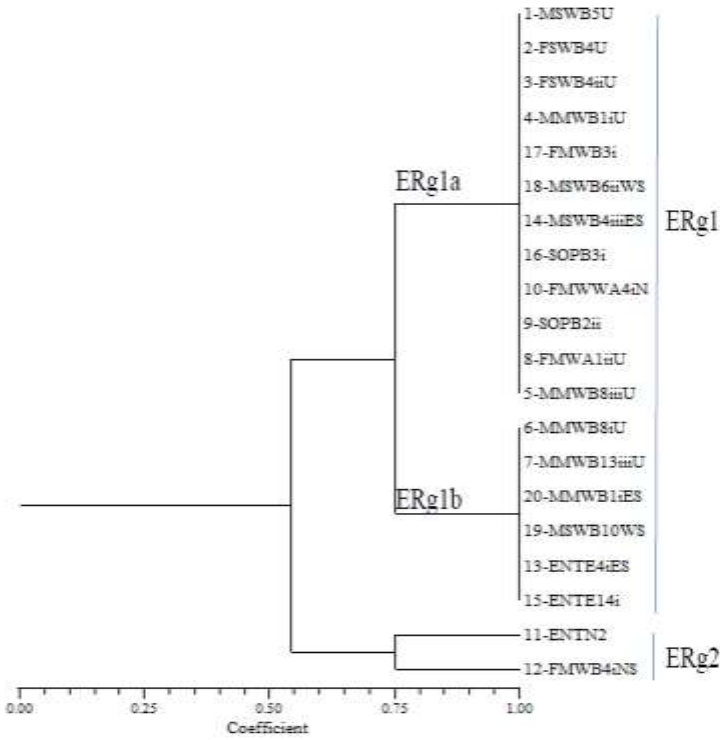

Figure 4b. Cluster E showing the reaction of 20 *S. aureus* isolates against three plant leaf extracts.

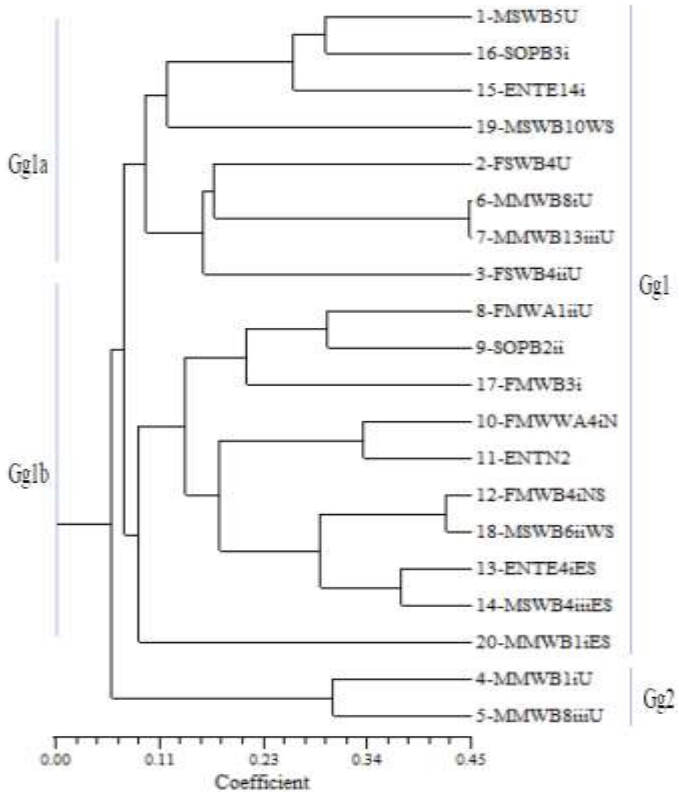

Figure 4c. Cluster G showing 20 *S. aureus* Isolates genotypes.

According to Adesida et al. (2007), identification of antibiotic resistant organism using molecular techniques such as 16s rRNA, random amplification of polymorphic DNA (RAPD) and plasmid profile causing infectious processes proves to be very essential for effective antimicrobial and supportive therapy.

Conclusion

By virtue of this analysis using molecular method (RAPD- PCR), it was possible to group isolates of similar genotypes with the same resistance behaviour to antibiotics and plants extract. Although major bands from RAPD reactions are highly reproducible, challenges like minor bands proves difficult to repeat due to random priming nature of PCR reaction and potential confounding effects associated with co-migration with other markers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Association of Official Analytical Chemists (AOAC) (1990). Official Methods of Analysis of the AOAC, 15th Edn. Washington, D.C. P 365.

Adesida SA, Abioye OA, Bamiro BS (2007). Associated risk factorsand pulsed field gel electrophoresis of nasal isolates of *Staphyloccous aureus* from medical students in a tertiary hospital in Lagos, Nigeria. Brazilian Journal of Infectious Diseases 11(1):63-69.

Adesino GO, Jibo S, Aggu VE. Ehinmidu JO (2011). Antibacterial activity of fresh juices of *Allium cepa* and *Zingiber ofiicinale* against multidrug resistant bacteria. International Journal of Pharmacy and Biological Sciences 2:289-295.

Akinyemi KO, Oladapo O, Okwara C E, Ibe CC, Fasure KA (2005). Screening of crude extracts of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant *Staphylococcus aureus* activity. BMC Alternative Med. 5:6-13.

Akpan EJ, Usoh IF (2004). Phytochemical screening and effect of aqueous root extract of Raphiahookeri (raffia palm) on metabolic clearance rate of ethanol in rabbits. Biochemistry 16(l):37-42.

Amadi ES, Nwofor GE, Ogbu O, Ayogu TE, Ononiwu CE (2007). Resistance of *Staphylococcus aureus* to commonly used antibiotic obtained from Different sources in Abakaliki. African Journal of Science and Technology 8(1):1728-1739.

Ankri S, Mirelman D (1999). Antimicrobial properties of allicin from garlic. Microbes and Infection 1:125 -129.

Ayoola GA, Coker HB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO (2008). Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in southwestern Nigeria. Tropical Journal of Pharmaceutical Research 7:1019-1024.

Baquero F, Coque TM, Canton R (2003). Antibiotics complexity and evolution. ASM News 69:547-552.

Chalita MK, Hofling-Lima AL, Paranhos A, Schor P, Belfort R (2004). Shifting trends in vitro antibiotic susceptibilities for common ocular isolates during a period of 15 years. American Journal of Ophthalmology 137(1):43-51.

Cheesbrough M (2003). Medical Laboratory Manual. Tropical Health

Technology, Low Priced Edition. Doddington, Cambridge shire, England. pp. 132-143.
Chethana GS, Hari Venkatesh KR, Giopinath SM (2013). Preliminary phytochemicals analysis of *Clerodendrum inerme*. International Research Journal of Pharmacy 4(5):208-209.
Chigbu, CO, Ezeronye OU (2003). Antibiotic resistant *Staphyloccous aureus* in Abia State of Nigeria. African Journal of Biotechnology 2(10):374-378.
Clinical and Laboratory Standards Institute (CLSI) (2012). Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard, 7th ed., CLSI document M02-A11. 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.
Fluit AC, Visser MR, Schmitz FJ (2001). Molecular detection of antimicrobial resistance. Clinical Microbiology Reviews 14:836-871.
Harborne JB (1973). Phytochemical Methods. Chapman and Hall Ltd, London. pp. 149-188.
Jako E, Ari E, Ittzes P, Horvath A, Podani J (2009). BOOL-AN: A method for comparative sequence analysis and phylogenetic reconstruction. Molecular Phylogenetics and Evolution 52:887-897.
Johansen IS, Lundgren B, Sosnovskaja A, Thomsen VO (2003). Direct detection of multidrug-resistant *Mycobacterium tuberculosis* in clinical specimens in low- and high-incidence countries by line probe assay. Journal of Clinical Microbiology 41:4454-4456.
Khan R, Baeshen MN, Saini KS, Bora RS, Al-Hejin AM, Baeshen NA (2016). Antibacterial activities of *Rhazyastricta* leaf extracts against multidrug resistant human pathogens. Biotechnology & Biotechnological Equipment 30:1016-1025.
Levy SB, Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine 10:S122-S129.
Lino A, Deogracios O (2006). The *in-vitro* antibacterial activity of *Annona senegalensis*, *Securidacca longipendiculata* and *Steanotaenia araliacea*-Ugandan Medicinal plants. African Health Sciences 6(1):31-35.
Magaldi, S., Mata-Essayag, S. and Hartung, C. de Capriles (2004), Well diffusion for antifungal susceptibility testing. International Journal of Infectious Diseases 8 pp. 39-45.
Martinez JL, Baquero F (2002). Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. Clinical Microbiology Reviews15:647-679.
Mboso OE, Eyong EU, Odey MO, Osakwe E (2013). Comparative phytochemical screening of *Ereromastax speciosa* and *Ereromastax polysperma*. Journal of Natural Product and Plant Resources 3(2):37-41.
Ndip, RN, Ebah LME, Onile BA (1997). Antibiogram of *Staphylococcus aureus* from clinical syndromes in Ilorin, Nigeria. Journal of Medical Laboratory Science 6:24-26.
Obi CL, Iyiegbuniwe AE, Olukoya DK, Babalola C, Igunbor EO, Okonta A. (1996). Antibiogram and plasmids of *Staphylococcus aureus* and coagulase negative staphylococci isolated from different clinical sources. Central African Journal of Medicine 42(9):258-261.
Olajide R, Akinsoyinu AO, Babayemi OJ, Omojola AB, Abu AO, Afolabi KD (2011). Effect of processing on energy values, nutrient and anti-nutrient components of wild cocoyam *Colocasia esculenta (L) Scott*. Corm. Pakistan Journal of Nutrition 10(1):29-34.
Oluduro AO, Bakare MK, Omoboye OO, Dada CA, Olatunji CI (2011). Antibacterial effect of extracts of *Acalypha wilkesiana* on gastrointestinal tract pathogens and bacteria causing skin infections in neonates. Ife Journal of Science 13:2.
Onah JO, Akabue PI, Okide JF (2002). The kinetics of reversal of pre-sickled erithrocytes by the aqueous extract of *Cajanus cajan*. Phytotherapy Research. 16(8):748-750.
Onasanya A, Mignouna HD, Thottappilly G (2003). Genetic fingerprinting and phylogenetic diversity of isolates of *Staphylococcus aureus* from Nigeria. African Journal of Biotechnology 2:246-250.
Oyewole OI, Malomo SO, Adebayo JO (2008). Comparative studies on antisickling properties of thiocyanate, tellurite and hydroxyurea. Pakistan Journal of Medical Sciences 24(1):18-22.
Pant J, Rai SK (2007). Occurrence of *Staphyloccous aureus* in Hospital Environment and Staffs in Teaching Hospital in Katmandu, Nepal. Nepal Association for Medical Laboratory Science 8:72-73.
Paul MO, Aderibie DA, Sule CZ (1982). Antimicrobial sensitivity patterns of hospital and non-hospital strains of *Staphylococcus aureus* isolated from nasal carriers. Journal of Hygiene 89:253-260.
Singleton VL, Orthofer RL, Rosa M (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. 299:152.
Sneath PH, Sokal RR. (1973). The Principle and Practice of Numerical Classification. In: Numerical Taxonomy, Kennedy, D. and R.B. Park (Eds.). Freeman, San Francisco.
Valgas C, De Souza ME, Smania FA (2007). Screening methods to determine antibacterial activity of natural products. Brazilian. Journal of Microbiology 38:369-380.
Walson D (1989). Principles and Practice of Statistics in Biological Research, 2nd ed. ISBN 0-7167-1254-7, P 53.
Warren DK, Liao RS, Merz LR, Eveland M, Dunne WM Jr. (2004). Detection of methicillin-resistant *Staphylococcus aureus* directly from nasal swab specimens by a real-time PCR assay. Journal of Clinical Microbiology 42:5578-5581.

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Full Length Research Paper

Genetic diversity using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers in *Tinospora cordifolia* from the Rayalseema region in Andhra Pradesh

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Tinospora cordifolia **is a highly important medicinal plant in India, with a rich source of different secondary metabolites. In the present study, 12 samples of** ***T. cordifolia*** **(collected from different areas of Rayalseema region,Andhra Pradesh) were studied for genetic diversity using 15 random amplified polymorphic DNA (RAPD) and eight inter-simple sequence repeats (ISSR) markers. Out of them, 10 RAPD and 5 ISSR primers showed 122 polymorphic reproducible bands across the selected 12 samples of** ***T. cordifolia.*** **The average polymorphic index content (PIC) of RAPD and ISSR values was 0.27 to 0.41 and 0.17 to 0.41, respectively. The PIC with RAPD markers was the highest for primers OPV 17 (0.41) and OPW 17(0.41) followed by the primer OPB 20 (0.40). The PIC with ISSR markers was the highest for the primer 824 (0.41) followed by primer 867 (0.36). Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of genetic similarity indices grouped all the samples into two major clusters. Jacquard's coefficient of similarity varied from 0.51 to 0.77, indicating high levels of genetic variation across genotypes under study. The result of this study can be used for characterization of potential** ***T. cordifolia*** **genetic resources and their utilization as breeding materials, micropropagation and secondary metabolites screening and harvesting.** ***T. cordifolia,*** **being a medicinal plant, is very useful for discovery of new lead molecules from this plant for drug discovery and development studies.**

Key words: *Tinospora cordifolia*, genetic diversity, random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), unweighted pair group method with arithmetic mean (UPGMA).

INTRODUCTION

Tinospora cordifolia (Willd.), belonging to the family Menispermaceae is an important climber medicinal plant species found at an altitude of 300 m, extending from the Himalayas down to the southern part of Peninsular India. The family Menispermaceae comprises about 72 genus and 450 species found in the tropical low land. *Tinospora* is one of the important genus of this family, consisting of about 32 species distributed in tropical Africa,

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Madagascar, Asia to Australia and the Pacific Islands (Kubitzki et al., 1993; Mabberley, 1997). It is also found in China, Myanmar, Sri Lanka, Thailand, Philippines, Indonesia, Malaysia, Borneo, Vietnam, Bangladesh, Pakistan, North Africa, West Africa and South Africa (Singh et al*.,* 2003; Mia et al., 2009; Jain et al*.,* 2010). The wood is white, soft, and porous. Leaves are simple, alternate, exstipulate, long petiolate, chordate shaped, long thread-like aerial roots arising from the branches with small unisexual yellow or greenish yellow flowers (Anonymous; Wealth of India, 1976; Kirthikar and Basu 1975). Fruits are red, fleshy, with many drupelets on a thick stalk. Three major groups of compounds; protoberberine alkaloids, terpenoids and polysaccharides are considered as putative active constituents in *T. cordifolia* (Chintalwar et al., 1999; Bisset and Nwaiwu, 1983). The stem is bitter, stomachic, diuretic (Nayampalli et al., 1988), stimulates bile secretion, causes constipation, allays thirst, burning sensation, vomiting, enriches the blood and cures jaundice, *T. cordifolia* is extensively used in the Indian Ayurvedic system of medicine as a tonic and also exhibits anti-periodic, antispasmodic, anti-inflammatory anti-arthritic, anti-allergic activities and also is very popular for anti-diabetic treatment (Handique, 2014) and also used in many ayurvedic formulations for treatment of different infections (Shanti and Nelson, 2013).*T. cordifolia* shows good immunomodulatory response (Jitendra et al., 2014). The stem of *T. cordifolia* is used as medicine as reported in Ayurvedic Pharmacopoeia of India (Sivakumar et al., 2014). The extract of its stem is useful in skin diseases (Aiyer and Kolammal, 1963; Raghunathan and Mittra, 1982). The root and stem of *T. cordifolia* are prescribed in combination with other drugs as an antidote to snake bite and scorpion sting (Nadkarni and Nadkarni, 1976; Kirtikar and Basu, 1975; Zhao et al., 1991). The plant is very rigid and grows in almost all climates especially preferring warm climate. Planting is usually done during the rainy season (July to August).

The DNA profiling technique using Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR), is extensively used in plants for the study of biodiversity, gene identification, and identifying markers linked with traits of interest. Plant genetic stability study using molecular markers in medicinal plants like *Withania somnifera* and *Capparis deciduas* (Tyagi et al., 2010) and other plants like coffee species (Mishra et al., 2014), artichoke (Sharaf-Eldin et al., 2015), bamboo (Desai et al., 2015), rye grass (Ghariani et al., 2015), and lemongrass (Bishoyi et al., 2016) has been reported.

Irrespective of the plant source or age, RAPD patterns are consistent and very useful for germplasm characterization, estimation of genetic relatedness and conservation of plant genetic resources (Welsh and McClelland, 1990). ISSR markers are also PCR based. It involves amplification of DNA segments between two identical microsatellite repeat regions with high reproducibility due to the use of longer primers (16 to 25-mer), and its high annealing temperature (45 to 60°C) leads to higher stringency (Zietkiewicz et al*.,* 1994; Pradeep Reddy et al*.,* 2002). ISSR has been found to be a powerful, rapid, simple, economical and reproducible tool used in marker assisted selection, DNA fingerprinting, evolution and molecular ecology (Zhao et al., 2007; Gajera et al., 2010; Zhang and Dai, 2010). Based on the advantages of these two molecular markers, viz., RAPD and ISSRs, they were employed to assess the genetic variation at the molecular level in *T. cordifolia* (Willd.) collected from the Rayalaseema region of Andhra Pradesh.

T. cordifolia has been listed amongst 29 highly prioritized medicinal plants for agro climatic zone 8 (Rajasthan, Uttar Pradesh and Madhya Pradesh) of India as identified by the National Medicinal Plant Board (NMPB) New Delhi, Government of India. This plant has also been listed in 178 medicinal plant species in high volume trade by NMPB, New Delhi, India (National Medicinal Plant Board, 2014). Due to the enormous traditional, folk, ayurvedic, medicinal and phytochemical importance, this plant species are disappearing rapidly from their natural habitats and is becoming endangered. There are no reports on studies of molecular profiling with molecular markers reported from the Rayalseema region of Andhra Pradesh. Assessment of genetic diversity will help in identifying elite trees with superior traits and also help in correlation of genetic diversity with function. This will be useful for conserving morphological and biological diversity of a population.

MATERIALS AND METHODS

Plant collection

A total of 12 samples of *T. cordifolia* were collected from different places of the YSR Kadapa district and Kurnool districts of Rayalaseema region, Andhra Pradesh having an altitude ranging from 120 m to around 500 m, and latitude ranging from 14 to 15, and longitudes at 77 to 79 range as listed in Table 1 and they were used in the present study. The plants were authenticated in Department of Botany, Yogi Vemana University.

DNA extraction

Total genomic DNA was extracted from young leaves using modified CTAB method (Murray and Thompson, 1980). Approximately, 200 mg of young leaf tissue devoid of mid rib was taken and ground to fine powder in liquid nitrogen in a motor and pestle. The finely ground powder was then transferred to 2.0 ml Eppendorff and to this 1 ml of preheated 2X CTAB buffer containing 2% (w/v) CTAB, 1.4 M NaCl, 20 Mm EDTA, 100 mM Tris-HCl, pH 8.0 was added. To this mixture, 0.2% (v/v) -mercaptoethanol was added and the homogenate was incubated at 65°C for 90 min with constant gentle mixing. Later, the homogenate was cooled to room temperature and equal volume of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 10 min at 25°C. Supernatant was collected and to this 10 µl of RNase (10 mg/ml) was added and

Table 1. Collection site names with latitudes, longitudes, and altitudes of 12 samples of *Tinospora cordifolia*.

Name	Latitude	Longitude	Altitude (m)
Bhakrapet,YSR-KadapaDist	14.415	78.952	148
Kanampalli, Near Pulivendula, YSR-Kadapa Dist	14.346	78.209	361
Gandi, YSR-KadapaDist	14.321	78.448	240
D. Rangapuram , Kurnool Dist	15.263	77.814	480
Mantamampalli, YSR-KadapaDist	14.319	79.086	154
Pattikonda, Kurnool Dist	15.391	79.500	459
Pulivendula, YSR-KadapaDist	14.415	78.224	274
Chakrayapet, YSR-KadapaDist	14.263	78.494	248
Lakkireddypalli, YSR-KadapaDist	14.189	78.694	361
Kadapa, YSR-KadapaDist	14.484	78.824	132
Madhavaram, YSR-KadapaDist	14.414	78.982	138
Ontimitta, YSR-KadapaDist	14.397	79.024	119

incubated at 37°C for 30 min. To this, equal volumes of chloroform:isoamylalcohol (24:1) were added and extracted to remove any remains of proteins by centrifuging at 12,000 rpm for 10 min at 25°C (Eppendroff Centrifuge 5810-R). The supernatant was then transferred to fresh 1.5 ml Eppendorff, and to which 0.6 ml of ice cold isopropanol was added and incubated at -20°C for 30 min. The DNA is pelleted out by centrifuging at 12,000 rpm for 10 min, washed with 70% ethanol, air dried and dissolved in sterile double distilled water. Quantification of the genomic DNA was done by spectrophotometric measurement of UV absorbance at 260 nm (UV 1800 ShimadzuUV spectrophotometer). An aliquot of the DNA samples was diluted in sterile distilled water in a ratio of 1:1000 in a 1-ml cuvette and the optical density was determined at 260 and 280 against sterile distilled water as blank. The DNA concentration was calculated using the formula (Sambrook et al., 1989):

$$\text{DNA concentration } (\mu g/\mu l) = \frac{OD_{260} \text{ x } 60 \text{ (dilution factor in the above) x } 50}{1000}$$

The ratio of OD_{260} to OD_{280} was calculated to check the purity of DNA. DNA samples for analysis were diluted to 7.5 ng/µl for RAPD and ISSR analysis.

Analysis with RAPD markers

A set of 15 RAPD (decamer) primers was randomly selected and used for PCR amplification (Table 2). PCR reaction was performed in a volume of 20 µl reaction mix containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 5 pM of decamer primer, 250 µM each dNTP's (Biobasic, Canada) and 0.8 U of TaqDNA polymerase with 30 ng of genomic DNA. PCR amplification was performed on thermocycler (Eppendorf Master Cycler Pro AG 6321, Germany). With initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min, 37°C annealing temperature for 1 min, extension time of 1 min at 72°C, followed by final extension at 72°C for 10 min. The amplicons were resolved on 1.2% agarose gel in 0.5x TBE system with a constant voltage of 100V for 2 h. A 1 kb DNA ladder (Hi-Media, India) was included as a control size marker. The amplified DNA was stained with Ethidium bromide, observed and documented in Gel documentation unit (G Box Syngene, Synoptic, Ltd, U.K).

Analysis with ISSR markers

A total of eight ISSR primers were employed for PCR amplification (Table 3). PCR reaction was performed in a volume of 20 µl reaction mix containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 5 pM of decamer primer, 250 µM each dNTP's (Biobasic, Canada) and 0.8 U of Taq DNA polymerase with 30 ng of genomic DNA. PCR amplification was performed on thermocycler (Eppendorf Master Cycler Pro AG 6321, Germany). With initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension time of 1 min at 72°C following final extension at 72°C for 10 min. The amplicons were resolved on 1.2% agarose gel in 0.5x TBE system with a constant voltage of 100 V for 2 h. A 1 kb DNA ladder (Hi-Media Labs, India) was included as a control size marker. The DNA was stained with Ethidium bromide, observed and documented in Gel documentation unit (G Box Syngene, Synoptic, Ltd, U.K).

Data scoring and analysis

The reaction was carried out twice and the gel was checked for consistency of the polymorphic bands. The reproducible bands were scored in all the samples for 15 RAPD and 8 ISSR primers separately. Each amplified product was considered as a separate marker. The band profiles were scored only for distinct, reproducible bands after PCR for each of the RAPD and ISSR primers. The reproducible amplicons were scored as 1 for the presence of a band and 0 for its absence, respectively. Similarity matching (SM) DICE coefficient values for pair-wise comparison between accessions were calculated and a DICE coefficient matrix was constructed using the SIMQUAL subroutine. Jaccard's similarity coefficient values were calculated and dendrograms based on similarity coefficient values were generated using unweighted pair-group method with arithmetic means (UPGMA) by the NTSYSpc 2.02j software. The polymorphism information content (PIC) value was calculated using the formula: 2f(1-f), where f is the frequency of bands present and (1- f), is the frequency of bands absent.

RESULTS

Screening of 12 *T. cordifolia* samples from the

Table 2. RAPD primers used for the present study and their amplification status.

Primer name	Sequence	Annealing temperature (°C)	Amplification status
OPA-04	AATCGGGCTG	37	Amplified
OPA-17	GACCGCTTGT	37	Not Amplified
OPB-05	TGCGCCCTTC	37	Amplified
OPB-06	TGCTCTGCCC	37	Amplified
OPB-07	GGTGACGCAG	37	Not Amplified
OPB-20	GGACCCTTAC	37	Amplified
OPG-01	CTACGGAGGA	37	Not Amplified
OPG-14	GGATGAGACC	37	Amplified
OPO-04	AAGTCCGCTC	37	Not Amplified
OPO-06	CCACGGGAAG	37	Not Amplified
OPO-08	CCTCCAGTGT	37	Amplified
OPV-17	ACCGGCTTGT	37	Amplified
OPW-17	GTCCTGGGTT	37	Amplified
OPZ-06	GTGCCGTTCA	37	Amplified
OPAW-07	AGCCCCCAAG	37	Amplified

Table 3. ISSR primers used for the present study and their amplification status.

Primer name	Sequence	Annealing temperature (°C)	Amplification status
815	CTC TCT CTC TCT CTC TG	45	Not Amplified
819	GTG TGT GTG TGT GTG TA	45	Not Amplified
824	TCT CTC TCT CTC TCT CG	45	Amplified
825	ACA CAC ACA CAC ACA CT	45	Not Amplified
842	GAG AGA GAG AGA GAG AYG	45	Amplified
846	CAC ACA CAC ACA CAC ART	45	Amplified
851	GTG TGT GTG TGT GTG TYG	45	Amplified
859	TGT GTG TGT GTG TGT GRC	45	Not Amplified
867	GGC GGCGGCGGCGGCGGC	45	Amplified
870	TGC TGCTGCTGCTGCTGC	45	Not Amplified

Rayalseema region with 15 RAPD and 8 ISSR markers, showed amplified amplicons with 10 RAPD primers and 5 ISSR primers. RAPD and ISSR markers showed 55 and 54% of polymorphism, respectively. These decamer RAPD primers OPB-20 and OPB-5 gave minimum of 4 amplicons and maximum of 12 amplicons, respectively each ranging from 300 to 1100 bp. From the total of 8 ISSR primers tested, five showed amplification. These five ISSR primers were able to amplify a minimum of four to eleven amplicons across the accessions with an average PIC value of 0.17 (846) to 0.41 (824) among the accessions (Table 4). The PCR amplified products of RAPD and ISSR primers are shown in Tables 2 and 3. The amplified product ranged between 0.35 and 1.8 kb in size for RAPD's markers (Figure 3), while with ISSR primers the amplified product size ranged between 0.5 and 2 kb (Figure 4), respectively. In this study, the number of alleles generated for individual loci varied from four to twelve, hence explaining the PIC range between 0.27 and 0.41 with RAPD markers and 0.17 to 0.41 with ISSR markers, respectively.

A total of 122 polymorphic bands were scored from 10 RAPD and 5 ISSR primers. A dendrogram was constructed to reveal the genetic relationships among the *T. cordifolia* (Figure 1). The UPGMA-based dendrogram showed that, all of the 12 collections could be classified into two groups based on the 122 polymorphic amplicons between the samples. The genetic distances among 12 genotypes are shown in Table 4. The similarity values in terms of genetic distance ranged from 0.51 to 0.77. A dendrogram generated by cluster analysis (UPGMA method) based on the 122 polymorphic amplicons revealed that the total 12 collections were separable into two major clusters, Cluster-I and Cluster-II (Figure 2). Cluster-I includes only one collection made from the region of Kanampalli near Pulivendula of YSR-Kadapa

Table 4. Degree of polymorphism and PIC for RAPD and ISSR primers in 12 samples of *Tinospora cordifolia*.

Marker type and name	Total amplicons	Polymorphic amplicons	PIC
RAPD			
OPA 4	10	10	0.34
OPB 6	9	9	0.36
OPA W7	6	6	0.36
OPB 20	4	4	0.40
OPB 5	12	12	0.39
OPG 14	10	10	0.39
OPO 8	5	5	0.33
OPV 17	5	5	0.41
OPW 17	8	8	0.41
OPZ 6	8	7	0.27
ISSR			
824	11	11	0.41
846	11	5	0.17
851	10	8	0.25
867	9	8	0.36
842	4	4	0.33

district, while the rest 11 collections are grouped into Cluster-II. These 11 collections from the Cluster-II were grouped into two groups that include eight collections into Cluster-IIA while three collections in Cluster-IIB. The same has been reflected in the similarity table, where the similarity coefficient of the sample from Kanampalli is distinct from others with less similarity range of 0.39 to 0.63 compared to collection from Pattikonda and Kurnool with a high similarity range of 0.63 to 0.77 in comparison with other genotypes under study.

DISCUSSION

Though RAPD markers have the problem of reproducibility, until recently and till to date these are the proven markers in terms of cost effectiveness that were extensively used to study genetic diversity among the species of bacteria, fungi, insects, plants and animals. Genetic diversity of twelve populations of three mushroom species were evaluated using seven RAPD markers that were able to differentiate the percent variability within and among different populations of these mushroom species (Dwivedi et al., 2018). Sugarcane when genetic diversity was estimated contain similarity coefficient which ranged from 0.43 to 0.91 using thirty five RAPD markers while the ISSR primers could explain the similarity coefficient from 0.73 to 0.93 which is less compared to RAPD markers (Patel et al., 2018). Eighteen synthetic Elite-II hexaploid wheat lines were evaluated using RAPD and SSR based molecular markers and inferred genetic variability of these synthetic lines is due to the novel genetic background derived from *Aegilops tauschi* accessions used as parental lines (Ahmad, 2014). While twenty two RAPD markers were used to study genetic diversity and percent polymorphism of 20 cotton germplasm lines that can help in identification of novel parental lines for hybrid cotton development (Tidke et al., 2014). A total of 16 RAPD and six SSR markers were used to assess the genetic diversity within the *Jatropha curcas* lines of diverse origin along with two of its wild relatives and showed the effectiveness of these markers to differentiate the wild relatives from the cultivated lines of *Jatropa* (Reddi et al., 2016). Wang et al., (2016) effectively utilized 100 RAPD primers and evaluated population structure of ginseng (*Panax ginseng* C.A. Meyer) collected from different regions and estimated the extent of gene flow between populations while Chandrakala et al., (2017) had shown that combine application of RAPD and ISSR markers can be effectively utilize to study the molecular genetic diversity in *Canthium parviflorum* Lam a medicinally important plant with wide range of applications. Among RAPD, molecular markers have been successfully used in studying the degree of genetic diversity between the *Morinda citrifolia* (noni) plants on the Asian continent. Singh et al. (2012) and Patel et al. (2014) have revealed high genetic diversity in *M. citrifolia* plants in different regions of India. This was probably due to the fact that region was being considered the center of origin of this species.

In this study, the results showed that the sub clusters formed by plants of different location in Rayalseema region showed the level and extent of genetic variation

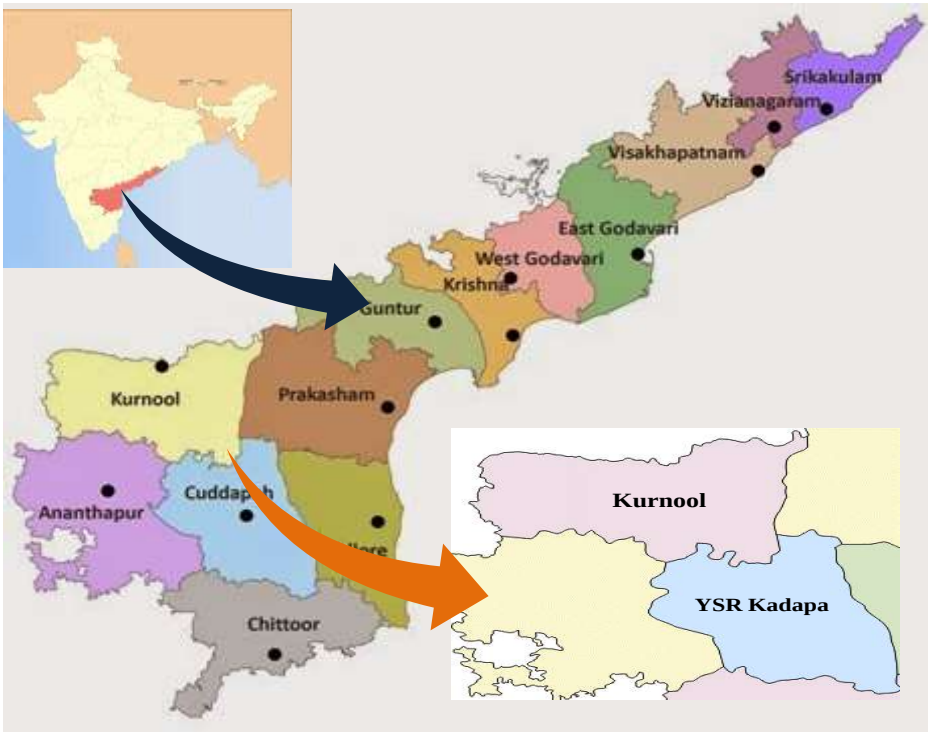

Figure 1. Geographical location of *Tinospora cordifolia,* where the samples were collected for the study.

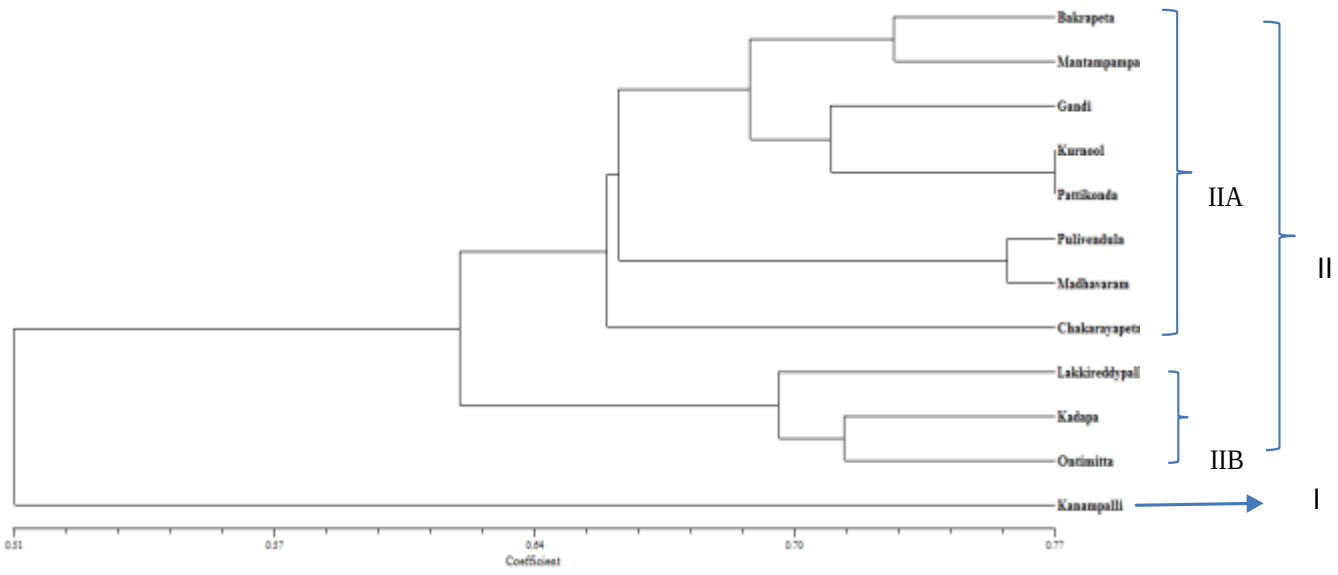

Figure 2. Phylogenetic relationships between 12 *Tinospora cordifolia* collections generated using NTSYS-pc program based on DICE similarity coefficients computed from data matrix with 122 informative polymorphic DNA bands generated from 10 RAPD and 5 ISSR markers.

present in them. The genetic distance similarity matrix among the 12 *T. cordifolia* collections is shown in Table 5. The similarity values in terms of genetic distance ranged from 0.39 to 0.77 between the samples from different places. Based on the molecular genetic studies and similarity index, samples from Kanampalli compared

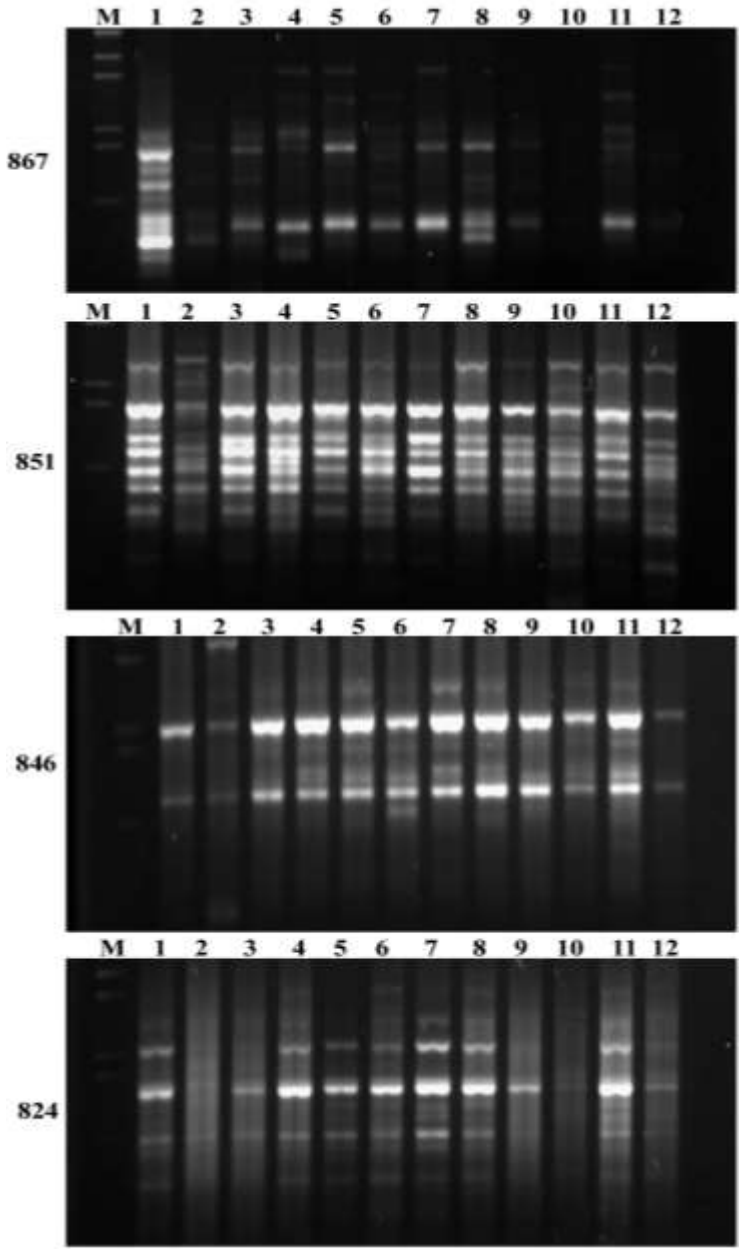

Figure 3. RAPD patterns of 12 samples of *Tinospora cordifolia*. M, Molecular weight Ladder; 1, Bakrapet YSR, Kadapa; 2, Kanampally, YSR, Kadapa District; 3, Gandi, YSR, Kadapa Dist; 4, D Rangapuram, Kurnool Dist; 5, Mantapampalli, YSR, Kadapa; 6, Pattikonda, Kurnool Dist; 7,Pulivendula, YSR, Kadapa; 8, Chakrayapet, YSR,KadapaDist; 9, Lakkireddypalli, YSR,Kadapa; 10, Kadapa, YSR KadapaDist;11, Madhavaram, YSR, Kadapa and 12, Ontimitta, YSR, Kadapa.

to any others were distant with an average similarity of 0.39 to 0.63 from other collections. Whereas collection from Kanampalli and Mantapampalli are more distant from each other with minimum similarity of only 0.39, collections from Kurnool and Pattikonda are much similar to each other with a similarity index of 0.77. It is also evident that from the dendrogram generated based on 122 number of amplicons, samples from Kanampalli of YSR-Kadapadistrict, Andhra Pradesh, India has turned out to be more distinct from the other 11 collections. It is justified by the fact that the geographical location of this place is much narrowly connected with the other places in the adjacent region, with a fertile valley surrounded by mountains, the population growing in this place is more

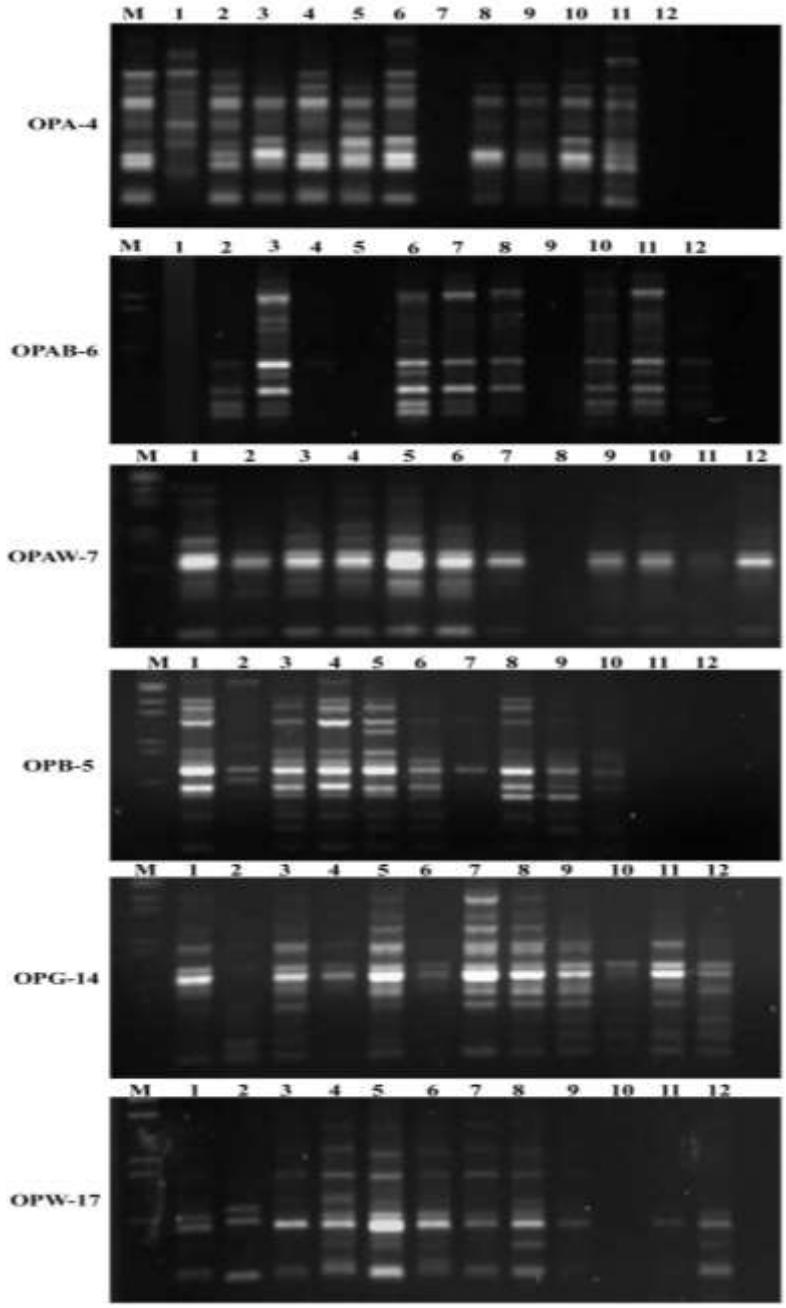

Figure 4. ISSR patterns of 12 samples of *Tinospora cordifolia*. M- Molecular weight Ladder; 1, Bakrapet, YSR, Kadapa; 2, Kanampally, YSR,Kadapa District; 3, Gandi, YSR, KadapaDist; 4, D Rangapuram, Kurnool Dist; 5, Mantapampalli, YSR, Kadapa; 6, Pattikonda, Kurnool Dist; 7, Pulivendula, YSR, Kadapa; 8, Chakrayapet, YSR,KadapaDist; 9, Lakkireddypalli, YSR,Kadapa; 10, Kadapa, YSR KadapaDist; 11, Madhavaram, YSR, Kadapa and 12, Ontimitta, YSR, Kadapa.

distinct with less genetic similarity index compared with other collections.

The polymorphism data generated can be used for plant breeding, crop improvement programs and also might be helpful in future strategies for evaluation of desired genotypes. More investigation on *Tinospora* genetic diversity is needed to evaluate more intra specific diversity analysis. Such studies on intra specific genetic diversity can contribute to the development of conservation strategies by identifying units for

Table 5. Jaccard's similarity coefficient matrix among 12 samples of *Tinospora cordifolia*.

Correlation	1	2	3	4	5	6	7	8	9	10	11	12
1	1.00											
2	0.52	1.00										
3	0.68	0.48	1.00									
4	0.67	0.44	0.68	1.00								
5	0.73	0.39	0.72	0.73	1.00							
6	0.66	0.46	0.75	0.77	0.70	1.00						
7	0.66	0.51	0.68	0.66	0.73	0.74	1.00					
8	0.63	0.47	0.63	0.68	0.68	0.67	0.70	1.00				
9	0.65	0.50	0.68	0.64	0.66	0.67	0.69	0.63	1.00			
10	0.57	0.63	0.64	0.55	0.51	0.58	0.63	0.51	0.69	1.00		
11	0.62	0.53	0.62	0.63	0.60	0.67	0.76	0.61	0.61	0.64	1.00	
12	0.64	0.63	0.58	0.66	0.55	0.63	0.68	0.58	0.71	0.72	0.69	1.00

1, Bakrapet; YSR, Kadapa; 2, Kanampally; YSR,Kadapa District; 3, Gandi; YSR, KadapaDist; 4, D Rangapuram; Kurnool Dist; 5, Mantapampalli; YSR, Kadapa; 6, Pattikonda; Kurnool Dist; 7, Pulivendula; YSR, Kadapa; 8, Chakrayapet; YSR,KadapaDist; 9, Lakkireddypalli; YSR,Kadapa; 10, Kadapa; YSR KadapaDist; 11 , Madhavaram; YSR, Kadapa; 12, Ontimitta; YSR, Kadapa.

conservation. John De Britto et al., (2010) studied and determined the genetic variability of *T. cordifolia* among the selected populations using RAPD markers and concluded the existence of low level of genetic variability in the species in a small geographical area among the different accessions of *T. cordifolia*. The number of polymorphic loci is 19 and the percentage of polymorphic loci is 46.34. The overall genetic distance and the genetic identity ranges from 0.1872 to 0.4555 and 0.6341 to 0.8293, respectively. The dendrogram showed three clusters between five populations and the studies revealed that wide heritable phenotypic and chemotypic variation in the collection of accessions might be due to qualitative genetic differences. Rana et al., (2012) demonstrated the genetic diversity analysis of *T. cordifolia* germplasm collected from northwestern Himalayan region of India.

The RAPD markers have also been employed in estimating genetic variability of various plants species. Molecular analysis in *Urginea indica* collected from different location of Karnataka was reported by Harini et al., (2008). Genetic diversity analysis in *Rauvolfia serpentine* and *Rauvolfia tetra phyla* L., using RAPD markers was carried out by Padmalatha and Prasad (2006, 2007). Ruan et al. (2008) analyzed the DNA molecular characters of *Centella asiatica* with RAPD techniques. The RAPD technique has been widely used both for studies on wild plants (Yeh et al., 1995; Khasa and Dancik, 1996; Manica-Cattani et al., 2009) and for studies on cultivated plants (Sharma and Dowsons, 1995; Yilmaz et al., 2012). In the present study, ISSR has been used as an effective molecular tool to calculate the genetic diversity on wild medicinal plant like *T. cordifolia*. Ahmed et al., (2006) utilized two molecular approaches, viz., 38 RAPD primers assay and restriction digestion of ITS1-5.8S-ITS2 Rdna (PCR-RFLP) in order to authenticate the results obtained by RAPD and PCR-RFLP to evaluate the genetic similarities between 40 different accessions belonging to three species (*T. cordifolia*, *Tinospora malabarica* and *Tinospora crispa*).

Three independent clones of each species were sequenced and phylogenetic relationship obtained from ITS sequences was found to be in agreement with RAPD data and inter and intra specific variation in the ITS region of *Tinospora* species that has been observed. The average proportion of polymorphic markers across primers was 95%, whereas restriction endonucleases showed 92% polymorphism. RAPD alone was found suitable for the species diversions. In contrast, PCR-RFLP showed bias in detecting exact species variation. There is limited information available for genus *Tinospora* where molecular markers like RAPD and ISSR were utilized for molecular genetic studies. The present analysis is based on ten RAPD and five ISSR markers which revealed significant genetic diversity among the *T. cordifolia* samples studied.

Conclusion

In this preliminary study of genetic diversity analysis which has not been reported earlier from this region, the existence of genetic diversity among a selected sample of *T. cordifolia* collected from various localities in Rayalseema region was reported. The results with RAPD showed that there is a need to take samples from wider populations situated at distant geographical locations to better assess their phylogenetic relationship so as to preserve their diversity for the future work. Identification of intra-population diversity also forms a very essential pre-requisite for the genetic diversity analysis. This is the first report of genetic diversity analysis in *T. cordifolia*

from the Rayalseema region in Andhra Pradesh.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Agro-techniques of Selected Medicinal Plants,Vol.II, National Medicinal Plants Board, Department of AYUSH, Ministry of Health and Family Welfare, Government of India, 2014. ISBN No – 978-81-909121-3-6. www.nmpb.nic.in.

Ahmed SM, Verma V, Qazi PH, Ganaie MM, Bakshi SK, Qazi GN (2006). Molecular phylogeny in Indian *Tinospora* species by DNA based molecular markers. Plant Systematics and Evolution 256(1-4):75-87.

Ahmad K (2014). RAPD and SSR based genetic diversity analysis of elite-2 set of synthetic hexaploid wheats. African Journal of Traditional, Complementary and Alternative Medicines 11(4):9-13.

Aiyer KN, Kolammal M (1963). Pharmacognosy of Ayurvedic Drugs, Series 1. The Central Research Institute, Trivandrum.

Anonymous (1976). The Wealth of India. A dictionary of Indian raw materials. Vol. X., C.S.I.R.f New Delhi, India. www.niscair.res.in

Bishoyi AK, Sharma A, Kavane A, Geetha KA (2016). Varietal discrimination and genetic variability analysis of Cymbopogon using RAPD and ISSR markers analysis. Applied Biochemistry and Biotechnology 179(4):659-670.

Bisset N, Nwaiwu (1983). Quaternary alkaloids of tinospora species. Planta Medica 48(8):275-279.

Chintalwar G, Jain A, Sipahimalani A, Banerji A, Sumariwalla P, Ramakrishnan R, Sainis K (1999). An immunologically active arabinogalactan from *Tinosporacordifolia*. Phytochemistry 52(6):1089-1093

Chandrakala S, Reddi KVNR, Sekhar AC, Reddy PCO, Mallikarjuna K (2017). *In vitro* propagation and genetic diversity analysis of Canthium parviflorum Lam by RAPD and ISSR markers. Annals of Plant Sciences 6(11):1775-1783.

Desai P, Gajera B, Mankad M, Shah S, Patel A, Patil G, Narayanan S, Kumar N (2015). Comparative assessment of genetic diversity among Indian bamboo genotypes using RAPD and ISSR markers. Molecular Biology Reports 42(8):1265-1273.

Dwivedi S, Singh S, Chauhan, UK, Tiwari MK (2018). Inter and intraspecific genetic diversity (RAPD) among three most frequent species of macrofungi (*Ganoderma lucidum*, *Leucoagricus* sp. And *Lentinus* sp.) of Tropical forest of Central India. Journal of Genetic Engineering and Biotechnology 16(1):133-141.

Ghariani S, Elazreg H, Chtourou-Ghorbel N, Chakroun M, Trifi-Farah N (2015). Genetic diversity analysis in Tunisian perennial ryegrass germplasm as estimated by RAPD, ISSR, and morpho-agronomical markers. Genetics and Molecular Research 14(4):18523-18533.

Gajera BB, Kumar N, Singh AS, Punvar BS, Ravi kiran R, Subhash N, Jadeja GC (2010). Assessment of genetic diversity in castor (*Ricinus communis L*.) using RAPD and ISSR markers. Industrial Crops and Products 32(3):491-498.

Handique PJ (2014). *In Vitro* Propagation and Medicinal Attributes of *Tinospora Cordifolia:* A Review. Austin Journal of Biotechnology and Bioengineering 1(5):5.

Harini SS, Leelambika L, Kameshwari S, Sathyanarayana N (2008). Optimization of DNA isolation and PCR-RAPD methods for molecular analysis of *Urginea indica* Kunth. International Journal of Integrative Biology 2(2):138-44.

Jain S, Sherlekar B, Barik R (2010). Evaluation of antioxidant potential of *Tinospora cordifolia* and *Tinospora sinensis*. International Journal of Pharmaceutical Sciences and Research 1(11):122-128.

Khasa PD, Dancik BP (1996). Rapid identification of white-Engelmann spruce species by RAPD markers. Theoretical and Applied Genetics 92(1):46-52.

Jitendra Mittal, Madan Mohan Sharma, Amla Batra (2014). *Tinospora cordifolia*: a multipurpose medicinal plant- A review. Journal of Medicinal Plants Studies 2(2):32-47.

John De Britto A, Leon Stephan Raj T, Petchimuthu K, Benjamin JeyaRathna, Kumar P, Mary Sujin R, Dharmar K(2010). Inter Population Genetic Variability in *Tinosporacordifolia*(wild.) Miers ex Hook. F. &Thoms .(*Menispermaceae*), through RAPD marker, Sciencia Acta Xaveriana 1(1):57-64.

Kirthikar KR, Basu BD (1975). Indian Medicinal Plants Vol-4. Bishen Singh Mahendra Pal Singh; Dehradun. https://archive.org/details/in.gov.ignca.2048/page/n5

Kubitzki K, Rohwer JG, Bittrich V (1993). The families and genera of vascular plants. Vol.2. Flowering plants, dicotyledons: Magnoliid, Hamamelid, and caryophyllid families. https://doi.org/10.1007/978-3-662-07255-4

Mabberley DJ (1997). The plant-book: a portable dictionary of the vascular plants. Cambridge University Press.

Manica-Cattani MF, Zacaria J, Pauletti G, Atti-Serafini L, Echeverrigaray S (2009). Genetic variation among South Brazilian accessions of *Lippia alba Mill*.(*Verbenaceae*) detected by ISSR and RAPD markers. Brazilian Journal of Biology 69(2):375-380.

Mia MM, Kadir MF, Hossan MS. Rahmatullah M (2009). Medicinal plants of the Garo tribe inhabiting the Madhupur forest region of Bangladesh. American Eurasian Journal of Sustainable Agriculture 3(2):165-171.

Mishra MK, Nishani S, Gowda M, Padmajyothi D, Suresh N, Sreenath H, Raghuramulu Y (2014). Genetic Diversity Among Ethiopian Coffee (Coffea Arabica L.) Collections Available In Indian Gene Bank Using Sequence Related Amplified Polymorphism Markers. Plant Breeding and Seed Science 70(1):29-40.

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8(19):4321-4326.

Nadkarni KM, Nadkarni AK (1976). Indian MateriaMedica, Vol 1. 3rd ed. Mumbai, M/S Popular Prakasan Pvt. Ltd. http://krishikosh.egranth.ac.in

Nayampalli SS, Ainapure SS, Samant BD (1988). A comparative study of diuretic effects of *Tinospora cordifolia* and hydrochloro-thiazide in rats and a preliminary phase I study in human volunteers. Journal of Postgraduate Medicine 34(4):233-236.

Patel P, Rajkumar BK, Parmar P, Shah R, Krishnamurthy R (2018). Assessment of genetic diversity in *Colletotrichum falcatum* Went accessions based on RAPD and ISSR markers. Journal of Genetic Engineering and Biotechnology 16(1):153-159.

Patel MN, Parmar LD, Parihar A, Singh AK, Sheikh WA (2014). A High-throughput DNA Extraction Protocol and its Utilization in Molecular Characterization of Noni (*Morinda citrifolia* L) Genotypes. Current Trends in Biotechnology and Pharmacy 8(2):166-174.

Padmalatha K, Prasad MN (2006). Genetic Diversity in *Rauvolfia tetraphylla Lf* using RAPD Markers. Journal of Plant Biotechnology 33(2):139-145.

Padmalatha K, Prasad MN (2007). Inter and intrapopulation genetic diversity of *Rauvolfia serpentina (L.) Benth*. ex Kurz, an endangered medicinal plant, by RAPD analysis. Medicinal and Aromatic Plant Science and Biotechnology 1(1):118-123.

Pradeep Reddy, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:9-17.

Reddi KVNR, Krishnasatya A, Ramesh P, Prasad LS, Narendra K, Reddy PCOR, Reddy CVCM, Sekhar AC (2016). Molecular genetic assessment of *Jatropha curcas* L.germplasm of diverse origin along with its wild relatives for various for growth and early establishment

related traits. International Journal of Pharma and Bio Sciences 7(4)(B):261-271.
Raghunathan K, Mitra RN (1982). Pharmacognosy of indigenous drugs, central council for research in Ayurveda and Siddha. New Delhi 2:687-695.
Rana V, Thakur K, Sood R, Sharma V, Sharma TR (2012). Genetic diversity analysis of *Tinospora cordifolia* germplasm collected from northwestern Himalayan region of India. Journal of genetics 91(1):99-103.
Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128(1):9-17.
Ruan Y, Mo RH, Li M, Huang LQ, Luo Y, Li XY, Zhou J, Wu YS (2008). Molecular characters of *Centella asiatica* found with RAPD technology. Zhongyaocai Journal of Chinese medicinal materials 31(7):970-973.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press.Cold Spring Harbor.New York. USA.
Shanti V, Nelson R (2013). Antibacterial activity of Tinospora cordifolia(Willd).Thoms on urinary tract pathogens. International Journal of Current Microbiology and Applied Sciences 2(6):190-194.
Sharaf-Eldin MA, Al-Tamimi A, Alam P, Elkholy SF, Jordan JR (2015). Genetic relatedness of artichoke (*Cynara scolymus* L.) hybrids using random amplified polymorphic DNA (RAPD) fingerprinting. Genetics and Molecular Research 14(4):18431-18439.
Sharma SK, Dawson IK, Waugh R (1995). Relationship among cultivated and wild lentils revealed by RAPD analysis. Theoretical and Applied Genetics 91(4):647-654.
Sivakumar V, Dhana Rajan MS, Mohamed Sadiq AM, Jayanthi M (2014). *In vitro* Micropropagation of Tinospora cordifolia (Willd.) Miers ex Hook. F. & Thoms - An Important Medicinal Plant. Journal of Pharmacognosy and Phytochemistry 3(2):5-10.
Singh DR, Shrawan S, Darick M, Anbananthan,V, Salim KM, Chaya K, Antia V (2012). Diversity of *Morinda citrifolia* L. in Andaman and Nicobar Islands (India) assessed through morphological and DNA markers. African Journal of Biotechnology 11(86):15214-15225.
Singh J, Sinha K, Sharma A, Mishra NP, Khanuja SP (2003). Traditional uses of *Tinosporacordifolia*(Guduchi). Journal of Medicinal and Aromatic Plant Science 25:748-751.
Tidke SA, Kiran S, Harke SN (2014). Analysis of genetic diversity in 20 cotton germplasm lines using Random Amplified Polymorphic DNA maker. Asian Journal of Plant Sciences 12(4-8):184-189.
Tyagi P, Khanduja S, Kothari SL (2010). *In vitro* culture of *Capparis decidua* and assessment of clonal fidelity of the regenerated plants. Biologia plantarum 54(1):126-130.
Wang S, Chen X, Han F, Li R, Li G, Zhao Y, Xu Y, Zhang L (2016). Genetic diversity and population structure of ginseng in China based on RAPD analysis. Open Life Sciences 11(1):387-390.
Welsh J, McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18(24):7213-7218.
Yeh FC, Chong DK, Yang RC (1995). RAPD variation within and among natural populations of trembling aspen (*Populustremuloide Michx*.)from Alberta. Journal of Heredity 86(6):454-460.
Yilmaz KU, Paydas-Kargi S, Dogan Y, Kafkas S (2012). Genetic diversity analysis based on ISSR, RAPD and SSR among Turkish Apricot Germplasms in Iran Caucasian eco-geographical group. Scientia Horticulturae 138(1):138-143.
Zhang LJ, Dai SL (2010). Genetic variation within and among populations of *Orychophragmus violaceus* (Cruciferae) in China as detected by ISSR analysis. Genetic Resources and Crop Evolution 57(1):55-64.
Zhao TF, Wang XK, Rimando AM, Che CT (1991). Folkloric medicinal plants: *Tinospora sagittata* var. cravaniana and *Mahonia bealei.* Planta Medica 57(5):505.
Zhao Y, Chen XY, Wang XR, Pian RQ (2007). ISSR analysis of genetic diversity among *Lespedeza bicolor* populations. Journal of Plant Genetic Resources 8:195-199.
Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20(2):176-83.

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