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African Journal of Biotechnology

Full Length Research Paper

Characteristics of *Bacillus subtilis* HNMY-13 and HNMY-15 strains in aflatoxin B_1 degradation and *Astragalus* bio-transformation

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***Astragalus* of traditional Chinese medicine (TCM) and *Bacillus subtilis* are extensively used in animal feed and for production of various fermented compounds. In order to observe the characteristics of HNMY-13 and HNMY-15 *B. subtilis* strains, this study focused on the optimization of parameters in liquid fermentation of the two strains in aflatoxin B_1 degradation, and *Astragalus* polysaccharide yield. The optimal parameters: degradation of aflatoxin B_1, the bacterial count, and the *Astragalus* polysaccharide yield analysis were examined in this study. It was shown that the percentage degradation of AFB_1 by HNMY-13 and HNMY-15 strains were 80.48 and 79.55% at 37°C, and 80.66% and 81.34 with pH 7.0. The percentage degradation by HNMY-13 was 88.07% after 48 h fermentation and by HNMY-15 was 84.81% after 60 h fermentation, respectively. The viable count of fermented sample was 8.8×10^8 and 7.6×10^8 CFU·mL^{-1} using HNMY-13 and HNMY-15 strains, respectively. The polysaccharide yielded by HNMY-13 was 4.93% at 48 h which is 1.7-fold higher than that of the control group, while polysaccharide yielded by HNMY-15 reached peak of 3.54% at 60 h. In conclusion, HNMY-13 and HNMY-15 of *B. subtilis* promote production of *Astragalus* polysaccharide in the process of liquid fermentation and can also degrade aflatoxin B_1 significantly; hence the combination could form a potential feed additive for animals.**

Key words: *Astragalus*, *Bacillus subtilis*, liquid fermentation, aflatoxin B1, polysaccharide.

INTRODUCTION

Bacillus spp. are dominant microflora in animal probiotics because they are Gram-positive, strict or facultative aerobe and endospore-forming bacteria (Chantawannakul et al., 2002). It was generally recognized that probiotics strains should be isolated from the gastrointestinal tracts (GIT) of their host. Such strains were claimed to have higher chance of survival and colonization in the intestine, which allow beneficial microbiota to thrive (Quigley, 2010). However, EU-authorized probiotics for animals were often not of intestinal origin (Nguyen et al., 2015).

According to previous studies, *Bacillus subtilis* could

biodegrade aflatoxins B_1 and ochratoxin A via biotransformation (Yu et al., 2015; Petchkongkaew et al., 2008). Thus, it could be directly applied in the feedstuffs and feeds (Gao et al., 2013). It also exhibited antimicrobial activity and high resistance to the simulated gut environment. In addition, *B. subtilis* ferments roasted soybean, wheat bran, fruit waste via solid state fermentation (SSF) for production of the novel fermented soybean, haloduracin and citric acid (Park et al., 2012; Danesh et al., 2013; Kumar et al., 2003).

Astragalus was used as supplementary forage supplement for livestock and poultry industry in China, because it contains polysaccharides, saponins, flavonoids, anthraquinones, alkaloids, amino acid, β-sitosterol and metallic elements (Ibrahim et al., 2013; Sun et al., 2013; Li et al., 2009). Recent literatures reported that crude extracts of *Astragalus* are observed to be anti-inflammatory (Kim et al., 2013), immunostimulant (Qin et al., 2012), antioxidative (Kim and Yang, 2005), and antiviral in isolated constituents (Kallon et al., 2013). *Astragalus* was fermented by lactic acid bacteria (LAB) using solid state fermentation (SSF) technology. This technology is superior to crude *Astragalus* (Sheih et al., 2011) due to its production of a wide range of antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins which could kill other microbes (Rolfe, 2000).

Even though literature has reported this based bio-transformation technology to determine the effects of *B. subtilis*, the use of *B. subtilis* on *Astragalus* effects has not been reported in literature. To the best of the authors' knowledge, this is the first investigation of *B. subtilis* from pig gastrointestinal tracts ferment *Astragalus*. It is significant to study the interaction between *Astragalus* and *B. subtilis*, producing in-feed antibiotic growth promoters (AGP) of livestock and poultry industry.

MATERIALS AND METHODS

Bacteria source and biochemistry experimentation

B. subtilis strains HNMY-13 and HNMY-15 were isolated from pigs' gastrointestinal tracts in Henan province of center of China. HNMY-13 and HNMY-15 isolates were grown on LB (Luria-Bertani) agar (Beijing Aoboxing Bio-tech company limited) at 37°C. Strains that were able to form spores on LB agar after 48 h were selected for further characterization. Further identification was achieved by performing the following tests: anaerobic test, Voges-Proskauer (VP) test, nitrate reduction test, amylohydrolysis, citrate utilization test, D-mannitol test, L-arabinose test, salt tolerance (7% NaCl in LB), growth at 37 and 42°C, and pH 5.7.

DNA extraction and 16S rRNA analysis

The bacterial isolates were grown overnight in LB agar at 37°C, then subjected to DNA extraction with bacterial genomic DNA miniprep kit (Suolaibao science and technology company, Beijing, China) according to the manufacturer's instructions. The genomic DNA is used as a template for PCR (94°C for 30 s, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 90 s and a final extension at 72°C for 10 min). The forward and reverse primers used to amplify the partial 16S rRNA sequence were 5'-CGCG GATC CCCA ATGA TATA GGTA AAAC-3' and 5'-CCGG AATT CTTA ATAG CTGT TACT TTGT-3', respectively. PCR reactions were performed in a triplicate 50 µL mixture containing 25 µL of PCR mix, 13.5 µL ultrapure water, 2 µL of primer 1 (10 µM), 2 µL of primer 2 (10 µM) and 7.5 µL of template DNA (0.2 ng/µL). Obtained sequences were compared with sequences in the GenBank non-redundant nucleotide database by BLAST analysis. Phylogeny was inferred from aligned nucleotide sequences of the 16S rRNA genes using MEGA6 software.

Optimization of fermentation parameters of *B. subtilis* HNMY-13 and HNMY-15 strains in aflatoxin B1 degradation

To determine the optimal parameters for degradation of aflatoxin B_1 by HNMY-13 and HNMY-15 strains, culture conditions for aflatoxin B_1 degradation were studied at different temperatures (25, 30, 32, 35, 37 and 40°C), pH values (5.0, 6.0, 7.0 and 8.0) and incubation periods (24, 36, 48, 60 and 72 h). Fermentation was carried out in a 500 mL Erlenmeyer flask containing 100 mL of medium inoculated with 100 µL of the spore suspension of the two strains (1.5×10^6 CFU·µL^{-1}) and incubated in an orbital shaker at 200 rpm. After the fermentation, 900 µL fermented liquid was collected from different treatment groups into a 1.5 mL sterile centrifuge tube and 100 µL AFB_1 (Aflatoxin B_1) standard substance (400 ng·mL^{-1}, Suolaibao science and technology company, Beijing, China) was added to each tube. A tube with only fermentation medium was set up as the control. Then, the AFB_1 residual content was determined using ELISA kits (Yisenbao biotechnology company, Beijing, China). The degradation rate can be described by the following formula: the percentage degradation of AFB_1 = [1-(content after processing AFB_1)/control group content]×100%.

Bacillus subtilis strains fermented *Astragalus*

Preparation of Astragalus concentrated solution

Astragalus was obtained from a central Chinese medicine market (Yuzhou, Henan, China). *Astragalus* (100 g) was taken and five-, three- and double-fold amount of water was added to boil three times, each time for 30 min. After boiling for three times, the filtrate was merged for 15 min by centrifugation (5000 ×g). The supernatant liquid was concentrated to 1 g·mL^{-1} by rotary evaporators. This suspension was later used as supplement for fermentation media.

Liquid fermentation

For the HNMY-13 and HNMY-15 strains, the LB medium was supplemented with 0.1, 0.3, 0.5, 0.7, 0.9 and 1 g·L^{-1} *Astragalus* concentrated liquid, respectively. Then they were inoculated with 10^6 CFU·g^{-1} of *B. subtilis* HNMY-13 and HNMY-15. The cultures were incubated at 37°C for 24 h under aerobic shaking condition. The samples were taken for further analysis.

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Table 1. Characterization of *Bacillus* spp. strains isolated from pig gastrointestinal tracts.

Strains	HNMY-13	HNMY-15
Sporulation (%)	100	98
anaerobic test	-	-
V-P test	+	+
nitrate reduction test	+	-
amylohydrolysis	-	+
gelatin liquefaction	-	-
citrate utilization test	-	+
D-mannitol test	-	+
L-arabinose test	-	-
LB NaCl (7.0%)	+	+
Growth pH 5.7	-	+
Growth at 37°C	+	+
Growth at 42°C	+	+

-Negative; +positive.

Microbiological analysis (total viable bacterial count)

Total viable bacterial counts were determined after the fermentation. The fermented liquid (1 mL) was mixed with 9 mL of sterilized physiological saline (0.85% NaCl). Serial dilutions were prepared in the sterilized physiological saline and 1 mL of liquid with appropriate concentration was poured in triplicate plates for the total viable bacterial count. Prepared test samples (1 mL) with the concentration of 10^{-7}, 10^{-8} and 10^{-9} dilution were transferred into sterile Petri dishes in triplicate. The warmed (45±2°C) sterile plate containing LB agar (15 mL) was mixed with the inoculums. Cultures were incubated aerobically at 37°C for 48 h. The colonies were then counted and expressed as logarithmic colony forming units per gram (log CFU·mL^{-1}) of the sample.

The *Astragalus* polysaccharides yield analysis

The fermented liquid was added to 95% ethanol (3 times volume) for 24 h, and then collected for centrifugation at 5000 ×g for 20 min. The precipitate was dried at 60°C and smashed into powder. The yield of polysaccharides in extracts was determined using the phenol-sulfuric acid method.

Statistical analysis

Data was expressed as mean±standard error of mean (S.E.M.). Statistical analysis was performed using GraphPad PRISM software version 6.0 (GraphPad Software, USA). A Two-way analysis of variance (ANOVA) with the step-down multiple-stage F post hoc test (Ryan-Einot-Gabriel-Welsch multiple F-test P=0.05) was performed to distinguish treatment mean differences.

RESULTS AND DISCUSSION

Biochemical identification of *B. subtilis* strains HNMY-13 and HNMY-15

Two spore-forming bacterial strains were isolated from GITs of pigs obtained from center of Henan province, China. Because the efficiency of sporulation is critical for industrial production of feed supplements with high yield and low cost, an initial screening was performed to assess sporulation strains and high sporulation efficiency (more than 95%). The two strains, named HNMY-13 and HNMY-15, were further characterized based on biochemical properties according to Berger's Manual of Systematic Bacteriology. Data shown in Table 1 indicated that HNMY-13 and HNMY-15 strains hydrolyze starch at a high level and metabolize glucose as determined by the Voges-Proskauer (VP) test. Under aerobic conditions, it was determined that the two strains grew at 37 and 42°C. HNMY-13 and HNMY-15 strains metabolized citrate and grew in 7.0% NaCl. However, HNMY-13 and HNMY-15 strains exhibited different results for nitrate reduction, amylohydrolysis, citrate utilization and D-mannitol test. Although biochemical identification was through traditional morphological and physiological method, this result showed that HNMY-13 and HNMY-15 strains had different growth characteristics based on the production of different metabolic products in the process of bacterial culturing.

PCR reaction and 16S rRNA analysis

The results of the PCR of the HNMY-13 and HNMY-15 strains are presented in Figure 1. The length of nucleotide sequences of the two strains was 1500 bp. The strains were assessed by 16S rRNA sequencing and BLAST analysis to determine species identity. Analysis of the phylogenetic relationship based on 16S rRNA sequences is presented in Figure 2. It also revealed that the HNMY-13 andHNMY-15 strains were closely related to *B. subtilis*. The use of 16S rRNA sequences to identify

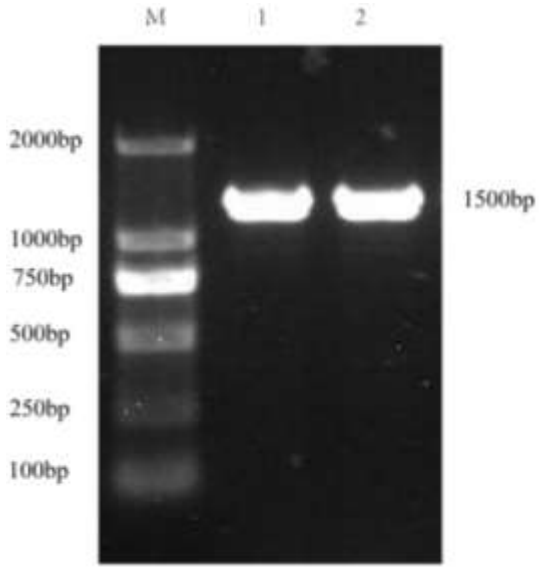

Figure 1. Agarose gel (1%) showing PCR products of HNMY-13 and HNMY-15 strains along with Marker DNA (2.0 kb). M: marker DL 2000; 1: HNMY-13 strain; 2: HNMY-15 strain.

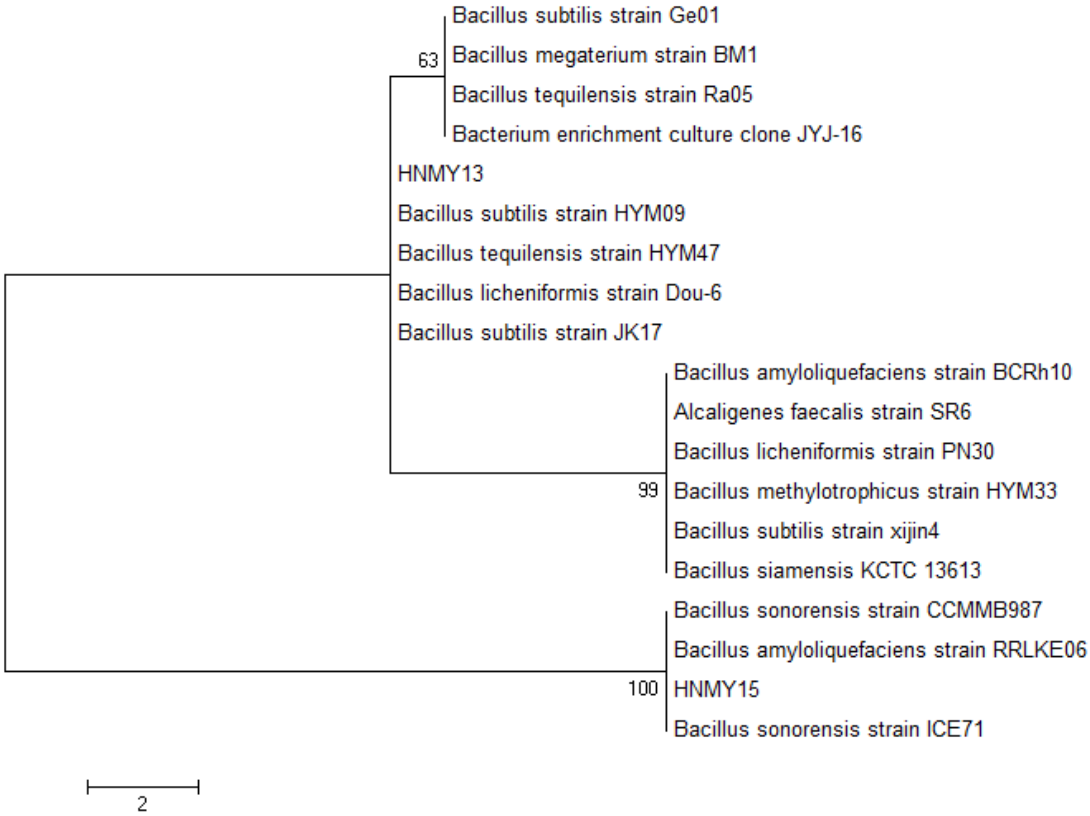

Figure 2. Phylogenetic relationship between the selected *Bacillus* strains. Dendrograms of strains based on 16s rRNA sequence alignment was obtained using MEGA6 software. Selected *Bacillus* strains are highlighted in bold and GenBank accession numbers are shown in brackets. Statistical (bootstrap) values and a scale bar representing evolutionary distance are shown. The 16s rRNA gene sequence of the *Bacillus* genus was used as the root of the phylogenetic tree.

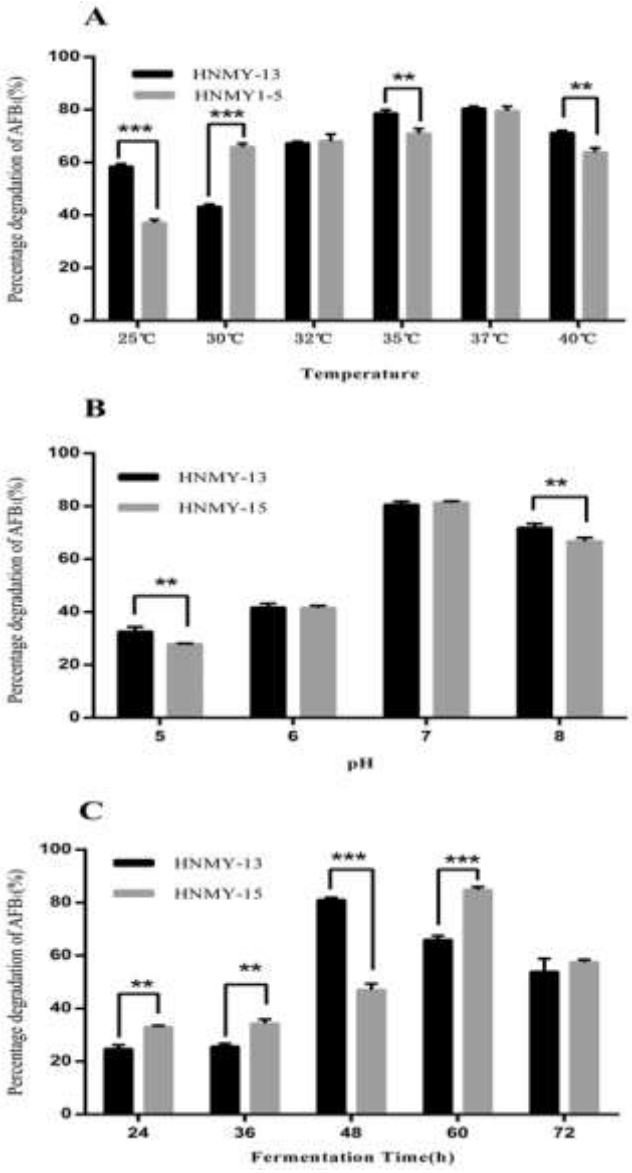

Figure 3. A: Percentage degradation of AFB_1 under different temperature (25, 30, 32, 35, 37 and 40°C) using HNMY-13 and HNMY-15 strains; B: Percentage degradation of AFB_1 at different pH values (5, 6, 7 and 8) using HNMY-13 and HNMY-15 strains; C: Percentage degradation of AFB_1 at different fermentation period (24, 36, 48, 60 and 72 h) using HNMY-13 and HNMY-15 strains. Bars with different asterisk indicates they significantly different from each other ($p<0.05$).

the species of bacteria has been successfully applied in modern microbiology, which could improve the accuracy of identification of the strain.

Optimization of HNMY-13 and HNMY-15 strains in aflatoxin B_1 degradation

The results of the optimal parameters are shown in Figure 3. The percentage degradation of AFB1 by HNMY-13 and HNMY-15 strains were respectively 80.48 and 79.55% at 37°C, and 80.66% and 81.34 with pH 7.0. The importance of temperature and pH value in the medium for aflatoxin B_1 degradation by different *B. subtilis* species has been reported by many investigators (Gao et al., 2013; Faraj et al., 1993). Percentage degradation by HNMY-13 was 88.07% after 48 h fermentation and by HNMY-15 was 84.81% after 60 h fermentation as shown

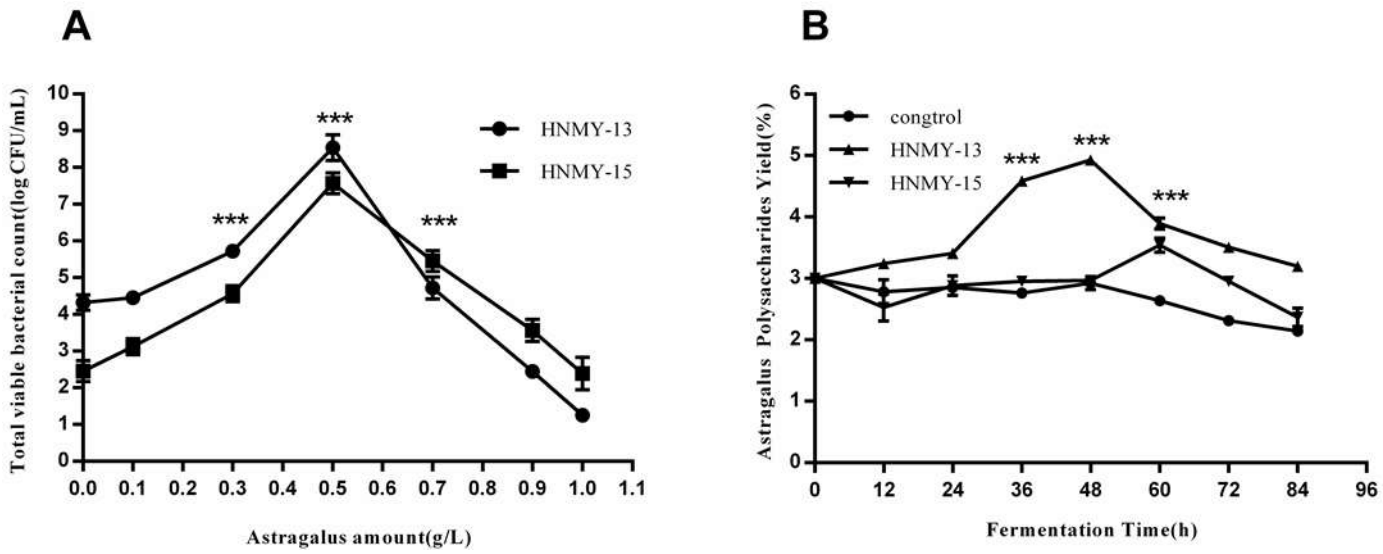

Figure 4. A: Microbial counts during fermentation with *B. subtilis* HNMY13 and HNMY15; B: The polysaccharide yield during fermentation with *B. subtilis* HNMY13 and HNMY15. Bars with different asterisk indicate they significantly differ from each other ($p<0.05$).

In Figure 3. The results revealed that different fermentation times had significant effect on HNMY-13 and HNMY-15 strains.

Microbial counts during fermentation

Microbiological monitoring of the fermented *Astragalus* co-inoculated with individual *B. subtilis* HNMY-13 and HNMY-15, respectively, was done. As shown in Figure 4A, the population of *B. subtilis* reached the peak in *Astragalus* amount at 0.5 g/L. The viable count of sample fermented was 8.8×10^8 CFU·mL^{-1} and 7.6×10^8 CFU·mL^{-1}, respectively. The result indicated that *Astragalus* might increase the population of *B. subtilis* under appropriate dosage. It was reported that *B. subtilis* natto-fermented *Astragalus* if fermented medium contained 10 g *Astragalus* (Hsu and Chiang, 2009). In this study, it was found that fermented medium containing 0.5 g could promote *B. subtilis* counts. This result revealed that *Astragalus* provided necessary polysaccharide, peptide and amino acid, such as glycine, valine, leucine, glutamic acid, tryptophan and isoleucine which were beneficial to *B. subtilis* reproduction. The result of this experiment was in line with previous studies (Gobbetti et al., 2010).

The polysaccharide yield analysis

This study presented fermented *Astragalus* by using *B. subtilis* HNMY-13 and HNMY-15 in liquid fermentation process, which was recognized as a potential bio-transformation process without causing severe environmental pollution. The *Astragalus* polysaccharide yield was higher with *B. subtilis* HNMY-13 and HNMY-15 fermentation as compared to the control group as shown in Figure 4B. Moreover, *Astragalus* polysaccharide yield by HNMY-13 was 4.93% at 48 h which was 1.7-fold higher than that of the control group, while polysaccharide yield by HNMY-15 reached peak at 3.54% after 60 h ($p<0.05$). The result showed that *Astragalus* polysaccharide yield changed significantly by *B. subtilis* fermentation. The result indicated that different bacteria had significant difference in effects on the yield of polysaccharide. In conclusion, HNMY-13 and HNMY-15 of *B. subtilis* promote production of *Astragalus* polysaccharide in the process of liquid fermentation and have the potential to be a feed additive.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ACKNOWLEDGEMENT

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Full Length Research Paper

The use of molecular markers in turtles: A technological prospecting

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The application of molecular markers stands out in studies on the genetic variability of animal and plant species. This tool contributes to studies on the history of some chelonian species, since it takes into consideration their longevity and the endangerment they are often exposed to. The aim of the current study is to perform a quantitative research to document and analyze the development of technological competencies based on the number of patents and scientific articles on the use of molecular markers in studies comprising chelonians. It was done using scientific articles from databases such as Science Direct, Web of Science, SCOPUS and CAPES thesis and dissertation bank, as well as patents recorded in databases such as INPI, WIPO and EPO. The results showed a larger number of scientific articles than patents. There was just one patent registration on the use of molecular markers in turtles; it was deposited in 2011 by a group of Chinese researchers. These indicators show an important scientific gap on this subject, which may be the basis of new studies aimed at better understanding and protecting this animal group.

Key words: Molecular markers, turtles, technological prospecting.

INTRODUCTION

The expansion of human activities worldwide has caused serious damages to the environment, namely: fragmentation of habitats, population decline and extinction of animal species. Many studies have been carried out in response to this “environmental crisis” as a way to gather information that can support the application and maintenance of effective measures aimed at protecting and managing wildlife resources.

Among the vertebrates, there is great concern about the conservation of turtles. According to Pough et al. (2008), in addition to the longevity observed in most species, these animals have low reproductive capacity to enable rapid population growth, given their low growth rates and the long period they need to reach sexual

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maturity and, thus, perpetuate the species. Data from the *International Union for Conservation of Nature* (IUCN) showed that their vulnerability to extinction endangerment rate reached 58.8%; this rate is much higher than that found in birds (13%), mammals (25%) and amphibians (41%). Páez et al. (2012) reported that more than 50% of the 322 turtle species that reached the 21st century are endangered. This situation becomes more severe if one takes into consideration, the continental (terrestrial and freshwater) species alone, which show 67% risk of extinction. According to Fantin (2008), such vulnerability results from anthropic activities, which devastate the natural habitat of these animals through predatory hunting during the breeding season and through the destruction of eggs.

The entire chelonian populations are endangered in some areas; almost all species in Southeastern Asia are endangered due to political and economic changes (Van Dijk et al., 2000). The situation is equally disturbing in China due to the use of these animals for dietary purposes and in several disease treatments (Pough et al., 2008).

Brazil holds 36 turtle species in several terrestrial and aquatic ecosystems. Five (5) out of this total are marine, 2 are terrestrial (red-footed tortoises) and 29 are freshwater species (Bérnils and Costa, 2012). Of the 20 Brazilian reptile species considered endangered or extinct, one in five chelonian species are endangered (Martins and Molina, 2008).

According to Bondioli (2009), it is essential to understand several aspects concerning endangered species such as taxonomic status, distribution area, as well as demographic and ecological issues. All these factors are crucial to develop species conservation proposals. Accordingly, Frankham et al. (2002) highlighted that low population density, in association with rapid population decline, contributes to faster allele loss, which hinders genetic variability and leads to deleterious allele fixation, adaptive potential reduction, and increased extinction likelihood. Cornuet and Luikart (1996) pointed out that identifying populations whose size was abruptly reduced is extremely important because these populations are more susceptible to extinction.

There has been significant advance in molecular genetics techniques in recent years, due to the use of molecular markers in different knowledge fields. These markers are useful and safe tools that can provide an accurate diagnosis. Comparative studies of the genetic variation described through molecular markers, as well as regional morphometric patterns, have focused on several wild species (Weksler et al., 2001; Vilela et al., 2010; Ross and Bouzat, 2014). The following molecular markers stand out among those used to detect genetic variability in DNA sequences: Restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), single nucleotide polymorphism (SNP), mitochondrial DNA (mt DNA) and microsatellites or short tandem repeats (STR) (Santos, 2011). They stand out because they show applicability in the development of different genetic-resource conservation strategies, since they provide complementary ecological and morphological data.

Table 1. Number of theses and dissertations found in CAPES database, according to individual keywords and groups of words, from October to November 2015.

Keywords	CAPES theses and dissertation
Molecular marker	1,396
Turtle	612
Chelonian	71
Molecular marker and turtle	0

Molecular markers provide a better understanding of the genetic diversity and demographic relations of faunal species in order to set conservation strategies. The aim of the current study was to document scientific information found in Brazilian, American and European databases by analyzing the development of technological competencies translated through the number of thesis, dissertations, articles and patents addressing the use of molecular markers in turtles.

MATERIALS AND METHODS

The herein presented manuscript is an exploratory documentary study of quantitative approach on technological prospecting, and it was carried out from October to November 2015. The thesis and dissertation bank belonging to the Coordination for the Improvement of Higher Education Personnel (CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) was used to search for information, whereas databases such as *Science Direct*, *Web of Science* and *SCOPUS* were used to search for scientific articles. Databases belonging to institutions such as the Brazilian National Institute of Industrial Property (INPI - Instituto Nacional de Propriedade Industrial), the European Patent Office (EPO) and the World Intellectual Property Organization (WIPO) were used to obtain information on the content of patents.

The keywords used in the search were *marcador molecular* (molecular marker), *tartaruga* (turtle), *quelônio* (chelonian), *marcador molecular e tartaruga* (molecular* and marker* and turtle*).

RESULTS AND DISCUSSION

Thesis, dissertations and scientific articles

Table 1 shows the number of thesis and dissertations deposited at CAPES database. The substantial number of studies on molecular markers may be attributed to the progress in molecular genetics and the use of its tools, as well as its several applications. These applications extend from animal and plant breeding to phylogenetic

Table 2. Number of scientific articles found in databases such as “Science Direct, Web of Science and SCOPUS”, according to individual keywords and group of words, from October to November 2015.

Keywords	Science Direct	Web of Science	SCOPUS
Molecular marker	593,630	300,512	160,194
Turtle	32,743	31,914	18,229
Chelonian	1,660	850	1,219
Molecular marker and turtle	2,753	205	110

Table 3. Number of patents per individual keyword, truncation and group of words searched in databases such as INPI, EPO and WIPO, from October to November 2015.

Keywords	INPI		EPO	WIPO
	Title	Abstract	Title or abstract	All fields
Marcador* molecular* or molecular marker*	1596	4886	5190	5058
Tartaruga* or turtle*	13	08	3686	3530
Quelônio* or chelonian*	01	01	13	08
Marcador* molecular* and tartaruga*/molecular* and marker* and turtle*	0	0	2	04

and population studies, in addition to its use in disease detection. According to Rosa and Paiva (2009), such progress generated knowledge and increased the species identification capacity beyond the characterization of biodiversity in several Brazilian ecosystems and the assessment of inter- and intra-population genetic variability in wild and domestic animals of economic importance. It is shown that the descriptors resulted in a robust number of thesis and dissertations when they were individually used in the searches; however, they did not present results when they were used in association, which may indicate limitation in the use of these genetic techniques.

Table 2 shows the distribution of scientific articles indexed in international databases. Considerable number of scientific articles on “molecular markers” was shown, which overall encompassed studies on animals and plants, thus confirming the applicability of these markers to different research lines. However, this number significantly decreased when keywords such as “molecular markers and turtles/chelonians” were used together. Such results may be explained by the fact that many species were not yet studied through these techniques, which explained the small number of biological data. According to Rodrigues (2002), Brazil is an atypical country when it comes to species conservation, despite its size and biodiversity in comparison with the United States, Russia, Australia, China and Canada. This is because, unlike these countries, Brazil has few resources to conserve such species.

Patents

Patent is a temporary ownership title over an invention or utility model; this title is granted by the State to inventors or authors, or to other natural or legal persons having rights over creations (INPI, 2013). Each country has its own legislation and according to Article 8 of Brazilian Law 9.279/96, “inventions meeting requirements such as novelty, inventive activity and industrial application are patentable” (Brasil, 1996).

Table 3 shows the distribution of patent filing applications based on the analysis applied to the keywords and their combinations in databases such as INPI, EPO and WIPO. A robust number of patents were found in the databases as mentioned above when the keyword “molecular marker” was used, fact that confirmed that this tool is of scientific interest. However, only 13 patents were found in the field “title” and eight were found in the field “abstract” when the keyword “turtle” was used in the INPI database. It is noteworthy that only one patent was found in all databases when the keyword “chelonian” was used, whereas no result was found when the keywords were used in association.

The analysis done in the four databases showed that turtles constitute a group of scientific interest, due to a reasonable number of patent applications. However, the refinement of the search using the association of words “molecular markers for/in turtles” significantly reduced the number of patent filings: only two were found in EPO and four were found in WIPO, which showed a gap in the registration of patents concerning this animal group. This issue is partly derived from the patenting legislation, which differs from country to country. Patents on living things and biological materials remain forbidden in Brazil. Bill n. 4961/2005, which is related to the access to genetic heritage, is in progress in the National Congress. In addition, Brazil has little participation in the

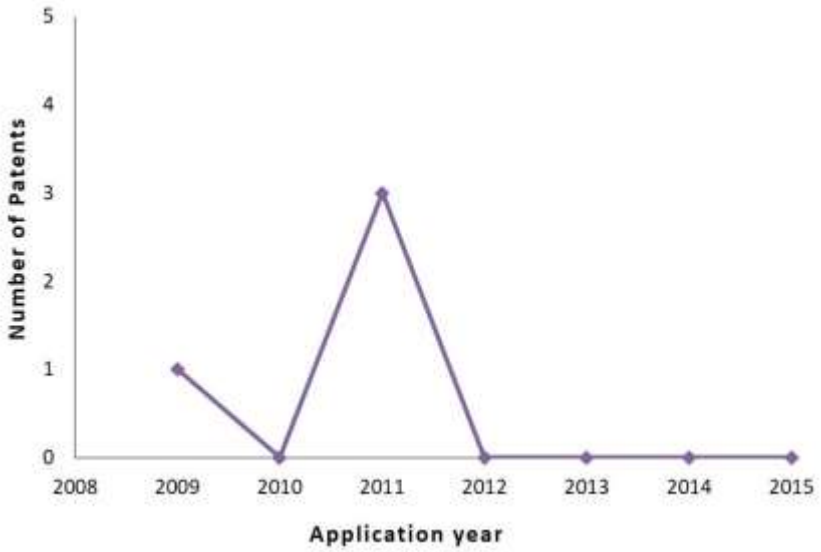

Figure 1. Annual evolution of patent filing based on the studied technology.

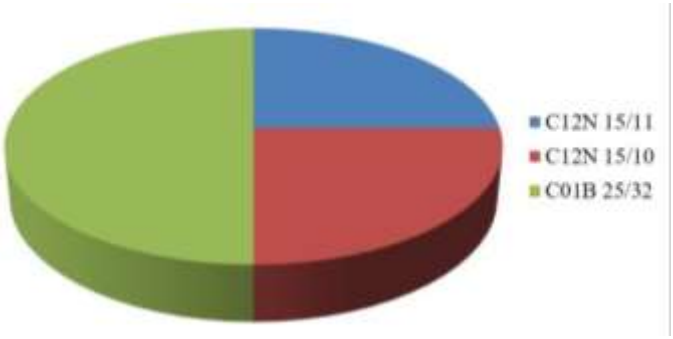

Figure 2. Patent application distribution according to IPC.

biotechnology segment. According to the World Intellectual Property Organization's Annual Report to the United Nations (UN) in 2014, Brazil occupies the penultimate position in the ranking of valid patents. According to statistical data, the United States remains the country that mostly focuses on international patent registration. It is followed by China, whereas emerging states need to strongly encourage technological innovation (Freire, 2016).

Patents associated with terms "molecular marker" and "turtle" in EPO and WIPO

Table 3 shows only 2 patents in the European database (EPO) and 4 in WIPO when the combinations between molecular marker(s) and turtle(s) or between molecular and marker* and turtle* were applied. Figure 1 shows the annual evolution of patent filing. It is shown that the first patent application occurred in 2009, whereas the other applications occurred in 2011. There was a single patent registration concerning conservation actions towards the turtle species, *Chinemys reevesii* (CN 102146382 A). The invention was based on providing microsatellite-type molecular markers with availability for 10 new informative loci and loci-amplification methods. According to the inventors, the invention could be applied to studies of the genetic diversity of different geographic populations, as well as paternity identification studies.

Serafini et al. (2011) highlighted the International Patent Classification (IPC) as a valuable tool to speed up searches on patent databases. This classification (Patentes on Line, 2015) distributes patents according to the application. The documents found in the herein conducted search were analyzed according to the IPC (Figure 2); 2 patents referred to section C12N (C12N 15/10 and C12N 15/11) and the other 2, to section C01B.

The IPC section C12 (biochemistry, beer, alcohol, wine,

vinegar, microbiology, enzymology, genetic or mutation engineering) covers microorganisms or enzymes; their composition, propagation, preservation or the maintenance of microorganisms or tissue; genetic or mutation engineering; and culture media, which are framed in subclass C12N. The patent applications belonging to this subclass were deposited in C12N 15/10 (mutation or genetic engineering, DNA or RNA concerning genetic engineering, vectors such as plasmids or their isolation, preparation or purification; the use of their hosts; recombinant DNA technology *DNA or RNA fragments* or *modified forms thereof*), as well as C12N 15/11 (mutation or genetic engineering; DNA or RNA concerning genetic engineering, vectors such as plasmids or their isolation, preparation or purification; the use of their hosts, recombinant DNA technology *DNA or RNA fragments* or *modified forms thereof*).

Section C01 (inorganic chemistry) involves non-metallic elements and their compounds (CO1B) in the subclass; classification of C01B 25/32 refers to phosphorus, its phosphorus oxyacid compounds, and its phosphate salts. Two (2) patents are classified in this section under the same main inventor's name: Bruce A. Keizer.

Patents vs. scientific articles

Scientific articles and patents are, respectively, means of disseminating scientific and technological knowledge (Mueller and Perucchi, 2014). Analysis of the patent/ scientific article ratio showed disparity in the evolution of publications in comparison with patent deposits on the use of molecular markers in turtles. A more detailed analysis of these articles revealed that, from 2002 on, there was a significant number of researches on genetic characterization using molecular markers; they only varied in the used marker type, and choice changed according to the aim of each study. However, despite evolution in the development of these genetic tools, interest in using them in turtles mainly focused on conservation, evolutionary and biological studies involving chelonians, and it results in lack of direct economic interest in these markers that could justify protecting the developed technologies through patent application. Besides, the arguments on patenting of living beings mainly include the possibility of finding the cure for diseases not yet completely elucidated by science; thus, it remains a path to be covered by Brazilian science.

Conclusion

Technological prospecting data showed a significant number of scientific articles on the use of molecular markers to study animal and plant species. However, there was disproportion between the number of studies and patents specifically referring to chelonians; a single patent regarding the species, *C. reevesii* was deposited in 2011 by Chinese researchers. It is hoped that data obtained in the current study can be used as reference base for new research, and they may encourage the use of molecular tools to help in better understanding and protection of turtle species.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Cloning and expression of acyl homoserine lactone (AHL) lactonase genes of *Bacillus cereus* INT1c and *Bacillus thuringiensis* SGT3g in *Escherichia coli*

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Inhibition of virulence genes expression of phytopathogenic bacteria can be done by degrading the acyl-homoserine lactone (AHL) compounds using AHL-lactonase. In this study, the AHL-lactonase genes were cloned and expressed from *Bacillus cereus* INT1c and *Bacillus thuringiensis* SGT3g in *Escherichia coli*, in order to understand the characteristics and biocontrol mechanisms of their AHL-lactonase. Amplification using *aiiA* primers succeeded to get 800 bp DNA fragments of AHL-latonase genes having a complete open reading frame (ORF) and encoding 250 amino acids. AHL-lactonase genes of *B. cereus* INT1c and *B. thuringiensis* SGT3g were expressed in *E. coli* BL21 (DE3) plasmid with T7 lysozyme coding sequence (pLysS). AHL-lactonase of the isolates is classified as metallo-β-lactamase superfamily domain and the AHL-lactonase inhibited violacein production of *Chromobacterium violaceum*.

Key words: Acyl-homoserine lactone (AHL), lactonase, *aiiA* gene, cloning, *Bacillus thuringiensis*, *Bacillus cereus*.

INTRODUCTION

Quorum sensing is a communication mechanism between intracellular bacterial cells that depends on the density of cells and it has an important role in regulating gene expression such as the gene that is responsible for biofilm formation, virulence factors, antibiotics synthesis, sporulation, and bioluminescence (Galloway et al., 2011). Quorum sensing is mediated by an extracellular signaling molecule called autoinducer. Autoinducer molecules are detected and responded by their bacterial cells when the concentration in the environment is high. Bacteria produce autoinducers of various types and Gram-negative bacteria produce N-acyl homoserine lactone (AHL) autoinducer (Hentzer et al., 2002).

Mechanisms of quorum sensing are used by phytopathogenic bacteria to express their virulence genes when they infect their host plants (Soto et al.,

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2006). Strategy to control phytopathogenic bacteria generally uses pesticides and antimicrobial compounds. However, the continuous use of these compounds will cause development of the phytopathogenic bacterial resistant. The alternative way to control phytopathogenic bacteria is by using quorum quenching mechanisms.

Application of quorum quenching has benefit, because it can reduce the emergence of phytopathogenic bacterial resistant. Quorum quenching does not affect viability of the patogenic bacterial cells, but it inhibits the pathogenic bacterial virulence mechanisms (Hentzer and Givskov, 2003). Concentration of AHL molecules is the main factor affecting the virulence genes expression, such that degradation of AHL molecules is one of the quorum quenching strategy. The enzyme that has the ability to degrade AHL molecules is AHL-lactonase (Molina et al., 2003). The enzyme has the function to hydrolyse the lactone ring of AHL molecules (Dong et al., 2000; Pan et al., 2007).

Isolation and characterization of bacteria producing AHL-lactonase were carried out from soil samples and leaves of agricultural lands in Java, Indonesia. A total of 12 isolates have the ability to degrade AHL and the isolates having the best AHL-lactonase activity were *Bacillus cereus* INT1c and *Bacillus thuringiensis* SGT3g (Afiah, 2011). Moreover, the *B. thuringiensis* SGT3g is capable of degrading AHL signal of *Dickeya dadantii* and can reduce bacterial soft rot symptoms disease caused by *D. dadantii* without any growth inhibition of *D. dadantii* on orchid leaves and the activity of its AHL-lactonase has a wide range of pH and temperature (Sari et al., 2016). So the enzymes of these bacterial isolates have the potential application to be used as an alternative biopesticide to control phytopathogenic bacteria. However, information of the genes encoding the enzymes of these bacterial isolates has not been studied yet. Therefore, this study aimed to characterize the *aii*A genes of *B. cereus* INT1c and *B. thuringiensis* SGT3g by cloning and expressing the genes in *Escherichia coli.* This information will help us to understand the gene characters and biocontrol mechanisms of their AHL-lactonase enzymes.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial isolates of *B. cereus* INT1c, *B. thuringiensis* SGT3g, *Chromobacterium violaceum*, *E. coli* DH5α and *E. coli* BL21 (DE3) plasmid with T7 lysozyme coding sequence (pLysS) were grown in Luria Bertani (LB) broth. *C. violaceum* was grown at 30°C for 24 h, while the other bacteria were grown at 37°C.

DNA isolation and amplification of AHL-lactonase gene

Bacterial DNA isolation was performed using cetyl trimethylammonium bromide (CTAB) methods (Sambrook and Russell, 2001). The concentration and purity of the extracted DNA was determined using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). The *aiiA* gene amplification was performed using *aiiAF* primer (5'-GGG CAT ATG ACA GGA GTA AAG AAG CTT TAT TTC G-3 ') and *aiiAR* (5'-GGG GGA TCC AAC CTC CTC ATA AAG ATG ATG CTA T-3') (Dong et al., 2000). Polymerase chain reaction (PCR) was set up for 30 cycles with condition as follow: pre-denaturation at 94°C for 2 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and post extension at 72°C for 5 min.

Subcloning of AHL-lactonase gene

DNA fragments of PCR products were subcloned in pGEMT-easy (Promega, Madison, USA). Recombinant plasmid was transformed into competent cells of *E. coli* DH5α by heat shock method at 42°C for 45 s. The transformant cells were grown on LB agar medium containing 100 µg/mL ampicillin, 40 µg/mL isopropyl β-D-1-thiogalactopyranoside (IPTG), and 40 µg/mL X-Gal and incubated at 37°C for 24 h (Sambrook and Russell, 2001). Recombinant plasmid isolation was carried out by growing the bacteria in LB broth medium containing 100 µg/mL ampicillin and incubating at 37°C for 16 h. Recombinant plasmid was isolated using Plasmid Mini Kit High Speed Isolation (Geneaid, South Korea). The recombinant plasmid was digested by *Eco*R1 and incubated at 37°C overnight. The fragments were run on 1.5% agarose gel electrophoresis.

Confirmation of transformant cells was done with amplification of the recombinant plasmid using primer T7 (5'-TAA TAC GAC TCA CTA TAG GG-3 ') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3'). The PCR was performed for 30 cycles with condition as follow: pre-denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min, and post extension at 72°C for 10 min.

Sequence analysis of recombinant AHL-lactonase gene

Recombinant AHL-lactonase genes were sequenced. Before constructing a phylogenetic tree using MEGA 5.0 software (Tamura et al., 2011), the sequences were edited using a Bioedit software. Amino acid analysis was performed using BLAST-X program and Open Reading Frame (ORF) analysis was performed using the ORF finder at https://www.ncbi.nlm.nih.gov. Protein domain and putative three-dimensional structure of the enzyme were determined using protein model base software at http://prosite.expasy.org.

Expression of AHL-lactonase gene and bioassay

Recombinants of AHL-lactonase genes were digested using *Bam*H1 and *Nde*1, then the fragments were ligated into pET15b expression vector and transformed into *E. coli* BL21 (DE3) pLysS. Transformant colonies were grown in LB broth containing ampicillin (100 µg/mL) and incubated at 37°C for 2 to 3 h. Verification of recombinant plasmid was done by plasmid isolation and digestion using *Bam*H1 and *Nde*1.

Expression of *aiiA* genes was induced by 1 mM IPTG when the culture optical density was reached at 0.6 and 0.8 (λ=600 nm), then the cultures were incubated at 37°C for 2 to 3 h on a shaker. Non-recombinan *E. coli* and recombinant *E. coli* without induction by IPTG were used as controls. Bioassay of recombinant protein activity was carried out using *C. violaceum* as a biocontrol. As much as 120 µL recombinant, *E. coli* was added on a paper disc (1.3 cm in diameter) and placed on LB semi solid agar medium containing 1% *C. violaceum*. The cultures were incubated at 30°C for 24 to 48 h. Activity of protein recombinant was shown by opaque zone formation around the paper disc.

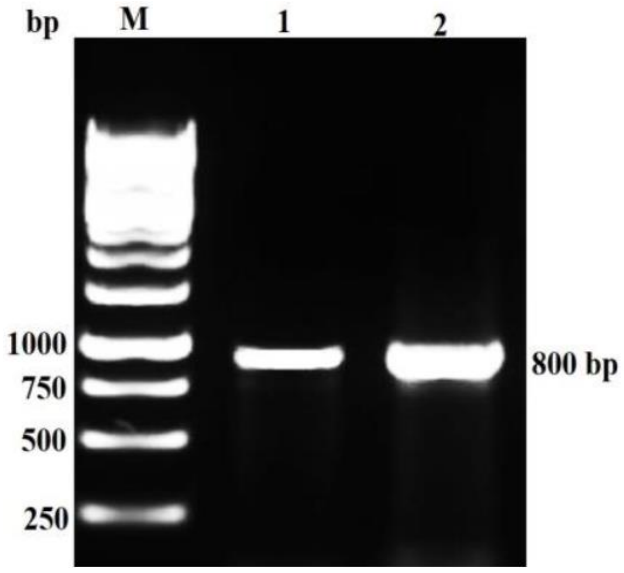

Figure 1. PCR products of *aiiA* genes on 1.5% agarose gel. M, 1 kb marker; lane 1, *B. cereus* INT1c; 2, *B. thuringiensis* SGT3g.

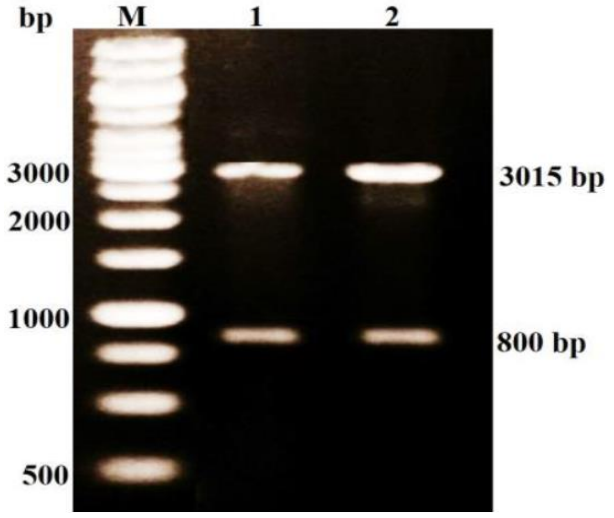

Figure 2. Restriction result of recombinant plasmid with *Eco*R1. M, 1 kb marker; lane 1, *B. cereus* INT1c; 2, *B. thuringiensis* SGT3g.

RESULTS

Insertion of AHL-lactonase genes in *E. coli* DH5α

Bacterial isolates used in this study were detected having the ability to inhibit violacein production of *C. violaceum*. These results indicate that *B. cereus* INT1c and *B. thuringiensis* SGT3g have a gene encoding AHL-lactonase. Amplification of AHL-lactonase gene from the isolates using *aiiA* primer succeeded in getting 800 bp of

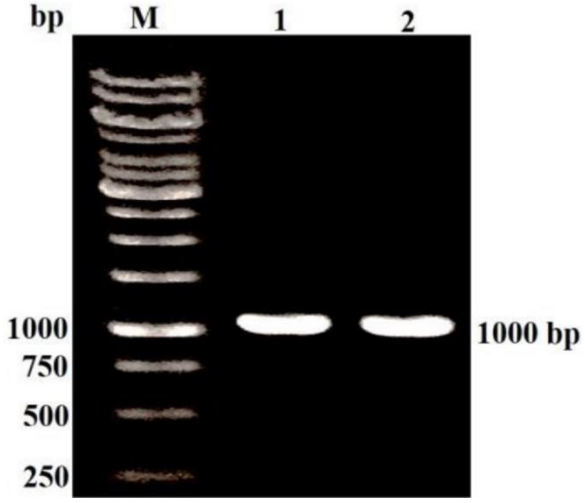

Figure 3. PCR product of recombinant plasmid in *E. coli* DH5α by T7 and SP6 primer. M, 1 kb marker; lane 1, *B. cereus* INT1c; 2, *B. thuringiensis* SGT3g.

DNA amplicont fragments (Figure 1). These fragments were subcloned in pGEMT-easy vector and they were propagated in *E. coli* DH5α. Confirmation of transformant cells digested using *Eco*R1 succeeded to separate the DNA inserts from pGEMT-easy vector. The fragment size of pGEMT-easy is 3015 bp and *aiiA* genes are 800 bp (Figure 2). Confirmation using T7 and SP6 primers could also amplify 1000 bp of DNA fragments which are total sequences of the DNA inserts and restriction sites in MCS (Figure 3).

Amino acid sequences analysis using BLAST-X indicated that *B. cereus* INT1c amino acid has 98% of maximum identity compared with *B. cereus* (accession number WP.000216573.1), while *B. thuringiensis* SGT3g has 99% of the maximum identity when compared with *B. thuringiensis* serovar *aizawai* (accession number AEY70474.1). Phylogenetic tree analysis based on amino acid sequences showed that the isolates also have a close relationship with other *Bacillus* groups such as *Bacillus amyloliquifaciens*, *Bacillus weihenstephanensis*, and *Bacillus thuringiensis* serovar *kim* (Figure 4).

ORF finder analysis showed that *B. cereus* INT1c and *B. thuringiensis* SGT3g have *aiiA* genes with complete sequences on orf2 which encode 250 amino acids. Conserved domains analysis of AHL-lactonase indicates that the enzymes belong to the metallo-β-lactamase superfamily domain. Three dimensional protein analysis showed that the enzymes have AHL-lactonase structure which is composed of αβ/βα folds and HXHXDH motif. The HXHXDH motif is composed of Zn^{2+} ions that bind to histidine and aspartate ligand (Figure 5).

aiiA expression and activity of AHL-lactonase

Digestion results by *Bam*H1 and *Nde*1 of recombinant

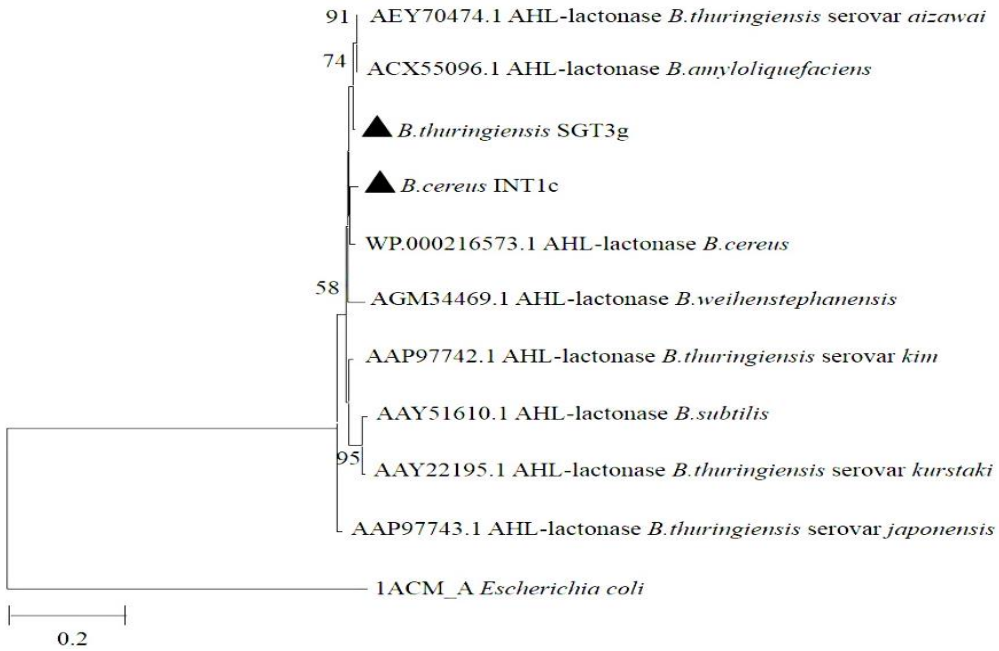

Figure 4. Phylogenetic tree of amino acid sequences of AHL-lactonase constructed using the neighbor-joining method.

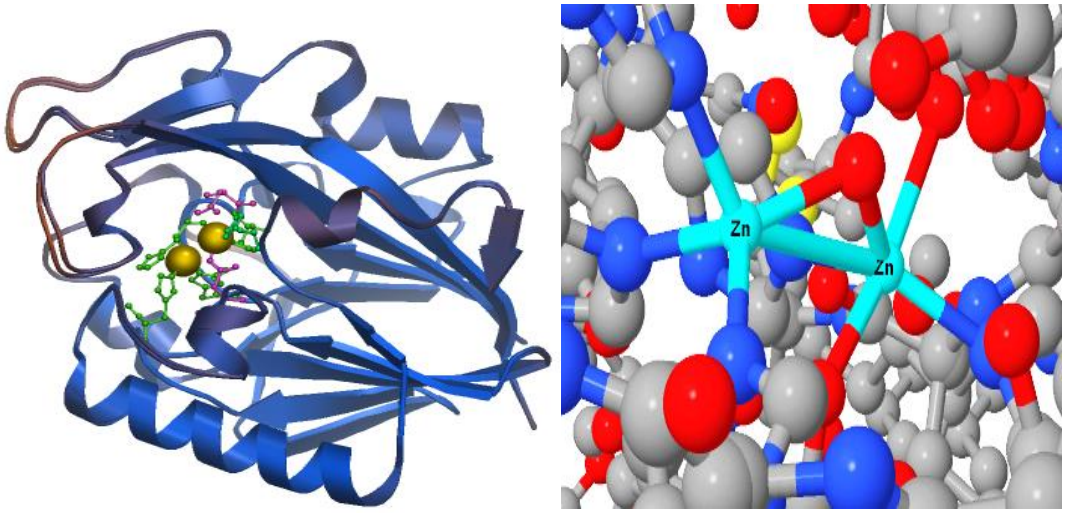

Figure 5. Predicted 3-dimensional protein structure of AHL-lactonase. Left, αβ/βα folds of AHL-lactonase; right, The HXHXDH motif is composed of Zn^{2+} ions that bind to histidine and aspartate ligands indicated by light blue, blue and red colours.

plasmids show the size of the pET15b DNA fragments (5708 bp) and the DNA inserts (800 bp) on 1.5% gel agarose (Figure 6). These results are evidence that the recombinant plasmids were propagated in the transformant cells. Addition of 1 mM IPTG in recombinant *E. coli* BL21 (DE3) pLySs can induce the expression of *aiiA* genes under control of T7 lac promoter. IPTG induction time affects AiiA proteins activity. Addition of 1 mM IPTG when the culture OD_{600} reached 0.6 can induce production of AiiA proteins with higher activity than that of the OD_{600} of 0.8. Recombinant *E. coli* with 0.6 OD_{600} has inhibitory activity to violacein production of *C. violaceum* that was characterized by opaque zone formation around a paper disc. However, when the

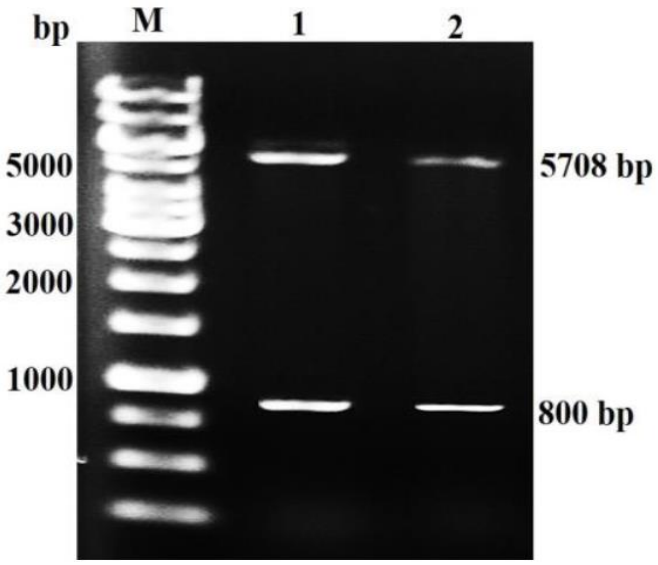

Figure 6. *Bam*H1 and *Nde*1 digestion results of recombinant plasmid in *E. coli* BL21 (DE3) pLysS. M, 1 kb marker; lane 1, *B. cereus* INT1c; 2, *B. thuringiensis* SGT3g.

culture OD_{600} was 0.8, IPTG induction did not show AHL degradation activity. And the recombinant *E. coli* BL21 and non-recombinant *E. coli* without IPTG induction as controls also showed the absence of AHL-lactonase activity (Figure 7).

DISCUSSION

AHL-degrading enzymes were found in proteobacteria groups and encoded by various genes such as *aiiA*, *aiiB*, *attM*, *ah1D*, and *aiiD* (Dong et al., 2000; Park et al., 2003; Carlier et al., 2003; Lin et al., 2003). Bacterial isolates of *B. cereus* INT1c and *B. thuringiensis* SGT3g were detected having high activity of AHL-lactonase. PCR ampification results indicate that the isolates have *aiiA* genes with 752 bp of DNA amplicont fragments. Several studies have also reported that AHL-lactonase of *Bacillus* group is encoded by *aiiA* genes (Dong et al., 2002; Lee et al., 2002; Anzhou et al., 2013). AHL-lactonase is an enzyme that can degrade AHL substrates with various acyl chain lengths (Cao et al., 2012). AHL-lactonase is commonly found in bacterial strains of *B. cereus* and *B. thuringiensis* (Dong et al., 2002; Lee et al., 2002; Flores et al., 2014), thus *aiiA* genes exploration from *Bacillus* group would help the biocontrol development of phytopathogenic bacteria that produce AHL compounds.

The *aiiA* primers used to amplify AHL-lactonase genes in this study are specific primers that amplify a region of the genes. Dong et al. (2000) also reported that the location of *aiiA* genes is not in plasmid, but in the chromosomal DNA. *B. thuringiensis* subsp. *kurstaki* strain B2 and *B. thuringiensis* subsp. *israelensis* strain B23 produce AHL-lactonase, although they have lost their plasmids.

B. cereus INT1c and *B. thuringiensis* SGT3g have similar AHL-lactonase genes sequences based on sequences alignment by MEGA 5.0. BLAST-X and phylogenetic tree analyses show that the isolates have close genetic relationship. Chromosome analysis still cannot distinguish genetic differences of both bacteria, whether they are different species or different varieties on the same species (Ivanova et al., 2003). This is due to the fact that *B. cereus* and *B. thuringiensis* have similarity on their phenotypes and genotypes (Oh et al., 2011). *B. thuringiensis* is distinguished from *B. cereus* because it has *cry* genes in their plasmids. The existence of these cry genes made of *B. thuringiensis* can produce parasporal crystals that are toxic to insects (Helgason et al., 2000).

AHL-lactonase of the isolates is classified as metallo-β-lactamase superfamily domain. This indicates that the enzymes have the same catalytic activity with the other metallo-β group-lactamase superfamily. Analysis of 3-dimensional protein structure also shows that the AHL-lactonase has HXHXDH motif and Zn^{2+} ions in the active site (Figure 4). Dong et al. (2000) reported that the HXHXDH motif is conserved in AiiA proteins. However, the motif is not owned by the AHL-lactonase *AidH* class (Gao et al., 2013). Zn^{2+} ions have an important role in the AHL-lactonase catalytic activity. The ions are coordinated by histidine and aspartate ligands to bind the substrates. The first Zn^{2+} ion is coordinated by His104, His106, His169 and bridges hydroxide ions (OH-), whereas the second Zn^{2+} ion is coordinated by Asp108, Asp191, His 109, His235 and bridges hydroxide ions (OH-) (Liu et

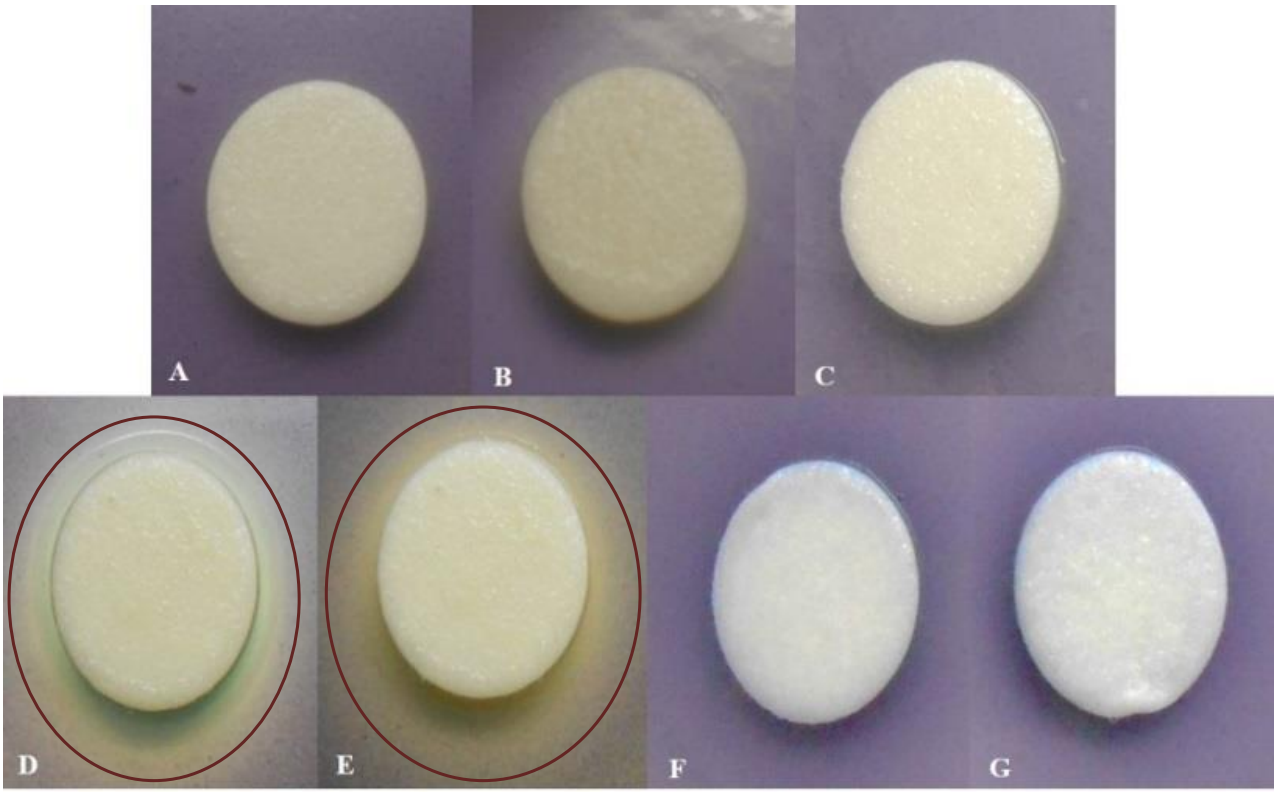

Figure 7. Bioassay of recombinant *E. coli* with *C. violaceum* as a biocontrol. A, *E. coli* BL21 (DE3) pLysS; B and C, *E. coli* BL21 (DE3) pLysS without IPTG induction; D, E, F and G, recombinant *E. coli* after induction with IPTG at optical density of 0.6 (D and E) and 0.8 (F and G). B, D, and F, recombinant *E. coli* with *aiiA* genes from *B. cereus* INT1c; C, E, and G, recombinant *E. coli* with *aiiA* genes from *B. thuringiensis* SGT3g. D and E show inhibition of violacein production indicated by circle lines.

al., 2005; Momb et al., 2008). Protein crystalic structure analysis of *B. cereus* AHL-lactonase shows that the histidine residues at HXHXDH motif are involved in Zn^{2+} ions binding, whereas aspartate residues are involved in catalytic reaction (Carfi et al., 1995).

Bioassay of AHL-lactonase activity shows that *E. coli* BL21 (DE3) pLysS recombinants inhibit violacein production in *C. violaceum*. *C. violaceum* uses a quorum sensing mechanism to form violacein pigment. The bacterium produces AHL compounds by synthesizing CviI signaling molecules (McClean et al., 1997; Duran and Menck, 2001; Stauff and Bassler, 2011). AHL-lactonase genes of the isolates were expressed after the cultures were induced by IPTG at the exponential phase. IPTG induction when OD_{600} culture reaches 0.6 can induce production of AiiA proteins with higher inhibition activity, however, the IPTG induction at optical density of 0.8 (OD_{600}) cannot induce production of aiiA proteins. Olaofe et al. (2010) reported that the recombinant *E. coli* induced using IPTG at the end of lag phase can inhibit bacterial cell growth, whereas induction in early exponential phase can increase the activity of the recombinant proteins about 3-fold higher compared to that of the end of exponential phase.

Recombinant *E. coli* generally produces insoluble proteins in aggregates form known as inclusion bodies. Bioassay using recombinant *E. coli* cultures shows AHL degradation activity. Park et al. (2003) reported that the addition of 1 mL recombinant *E. coli* cultures can effectively degrade a wide range AHL substrates. Expression of recombinant AHL-lactonase genes also can reduce virulence of *E. carotovora* on potatoes (Lee et al., 2002). Expression of recombinant proteins is influenced by various factors such as time of induction, long incubation after induction, incubation temperature, type of vectors and bacterial hosts (Dang et al., 2012; Fang et al., 2014; Picaud et al., 2007; Piubelli et al., 2013).

Conclusively, AHL-lactonase genes of *B. cereus* INT1c and *B. thuringiensis* SGT3g are expressed in *E. coli* BL21 cells (DE3) pLysS. The AiiA proteins produced by recombinant *E. coli* inhibit violacein production in *C. violaceum* by degrading AHL compounds. Expression of recombinant AHL-lactonase genes and their proteins activity are affected by IPTG induction time.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluating chemical composition of *Butia capitata* pulp among various populations and locations using multivariate analysis

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There are many native species from Cerrado that are used as food supply. However, their nutritional constitution is still unknown. The objective of this study was to evaluate and quantify the nutritional composition of *coquinho-azedo* fruits, obtained from different regions and populations in Northern Minas Gerais, using multivariate analysis. Ripe fruits of various *B. capitata* populations were obtained from four locations in Northern Minas Gerais. Fruit samples were analyzed for total acidity, total sugars, ascorbic acid, phenolic compounds, antioxidant activity, macro- and microelements. Results show that *Butia capitata* fruits are acidic given that values of titratable acidity ranged from 1.57 to 2.61, and Brix values ranged from 6.6° to 10.00°Bx. The total sugar content was relatively low with the lowest values observed in groups 4, 5 and 8 from Cristália (0.65, 0.75 and 0.78%, respectively). Ascorbic acid content also varied among populations and locations; the highest average (92.43) was observed in group 2 from Cristália and the highest value (821.63 mg/100 g fw) was observed in group 7 from Bonito de Minas. The highest antioxidant activity value (58.39%) was observed in group 7 from Cristália. Multivariate analysis grouped and separated populations that were genetically similar or dissimilar for ascorbic acid, antioxidant activity, total phenolic compounds and total sugars. The nutritional composition of *B. capitata* varied widely according to the sampled site and groups of plants. In general, the fruits contain high amounts of ascorbic acid, potassium and iron.

Key words: Arecaceae, ascorbic acid, antioxidant capacity, dendogram.

INTRODUCTION

Cerrado (Brazilian savannas) is the second largest biome in Brazil, and hosts a wide variety of native species (Klink and Machado, 2005), which are used for various purposes including the production of pharmaceuticals, cosmetics, food and feed (Klink and Machado, 2005; Souza et al., 2012). Native fruits of the Cerrado regions are used locally and they are consumed fresh or processed in jams, jellies, liqueurs and pastries (Almeida et al., 2011). They are very important for local economy because they help farmers and rural worker cooperatives

to generate income. Furthermore, many of these fruits are used in school meals for children.

Butia capitata Mart. Becc is a palm tree belonging to the Arecaceae family. It is native of Cerrado and found in the states of Goiás, Bahia and Minas Gerais (Lorenzi et al., 2010). This fruit is widely consumed in these regions; the fruits are constituted of a drupe oval yellowish, red or purplish epicarp and a yellow, succulent, and fibrous mesocarp (Moura et al., 2008). These are used for fresh consumption or pulp manufacturing, and are marketed regionally, or used for the production of jams, jellies, liqueurs, ice cream and popsicles (Faria et al., 2008).

Nutritional composition of *B. capitata*, similarly to many other native fruits of the Cerrado, has not been studied extensively. Further characterization and quantification of all constituents found in the pulp of this species is necessary. Faria et al. (2008) reported that the levels of ascorbic acid and potassium in pindo palm are higher than those observed in orange. Other species of the *Butia* genus showed high levels of total phenolic compounds and major carotenoids (Jacques et al., 2009).

Fruits are rich in vitamins, antioxidants, minerals and other constituents that are essential for the human body, because they participate in protein biosynthesis, cell regulation, and various processes in composite cells (Slavin and Lloyd, 2012). However, the concentration of these compounds may vary in among locations, because their production is influenced by environmental, edaphic, and genotypic factors (Asensi-Fabado and Munné-Bosch, 2011). Constituents in *B. capitata* may fluctuate significantly due to genotype, because it is a non-native domesticated species, as observed in other palm species. In *Phoenix dactylifera*, the concentration of phenolic compounds and total flavonoids varied among genotypes and locations (Biglari et al., 2008). The chemical composition of *Euterpe precatoria* juice varies due to location (Yuyama et al., 2011). Additionally, fruits of *Acrocomia aculeata* collected in warm regions had relatively higher amounts of reducing sugars, total acidity, and ascorbic acid (Sanjinez-Argandoña and Chuba, 2011).

Multivariate analysis allows an assessment of clustering individuals obtained from different locations. Cluster analysis allows the combination of individuals that are similar into separate groups (Tabachnick and Fidell, 2001). Therefore, it is a numerical methodology that proposes the classificatory structure or recognition of groups' existence. This method of analysis has been employed in various studies that aimed to characterize the bioactive compounds in fruits species, as *Mauritia flexuosa* and *Platonia insignis*. Dendogram clustering and principal component analysis allowed the formation of five distinct groups in a study of 18 fruit species (Barreto et al., 2009). Multivariate analysis was successfully used in *Averrhoa carambola* (Zainudin et al., 2014) and *Polygonum tinctorium* (Kim et al., 2012), as well.

The objective of this study was to evaluate and quantify the nutritional composition of *B. capitata* fruits collected from different populations and regions in Northern Minas Gerais using multivariate analysis.

MATERIALS AND METHODS

Plant material

Fruits of *B. capitata* were collected from four locations in the northern region of Minas Gerais: Abóboras, Mirabela, Bonito de Minas and Cristália. Twenty-four plants from each region were randomly sampled using a global positioning system (Garmin GPS III Plus). The population that had a median age of 25 years was native and economically exploited. Fruits were collected at maturity (yellow epicarp) and individually bagged and packed in polystyrene boxes with ice and transported to the laboratory. Fruits were washed with water and pulp was removed manually, using a stainless knife. Afterwards, it was placed in a plastic container and stored at -80°C in an ultra-freezer. Sub-samples were formed from the mixture of three plants and all analyses were performed three times. All tests were performed at the central laboratory of the Universidade Federal de Lavras (UFLA).

Physicochemical analysis

Physicochemical analysis of pH, titratable acidity (TA) and soluble solids (SS) was performed according to the analytical methods of the Association of Official Analytical Chemists (AOAC, 1992). The pH of pulps was determined by using a pH meter (Schott Handylab pH 11), and the determination of acidity was performed by titration with sodium hydroxide (NaOH) 0.1 N and phenolphthalein, as an indicator (Instituto Adolfo Lutz, 2008). Results were expressed as the percentage of citric acid. Soluble solids were identified by refractometry, using digital refractometer (ATAGO PR-100), previously calibrated with distilled water. Results were expressed in degrees Brix (°Bx). The ratio of soluble solids to titratable acidity (SS/TA) was also computed.

Mineral analysis

Samples of dry and degreased pulp were used for mineral analysis. Each pulp sample (5 g) was ground using stainless steel knives. Knife mill was cleaned after each sample to avoid cross contamination. Minerals such as phosphorus, potassium, magnesium, manganese, copper, zinc and iron were analyzed. Mineral content was determined as described by Malavolta et al. (1997), using atomic absorption spectrometer (Varian SpectrAA 110), calibrated to specific conditions of wavelength, slit and gas mixture for each element. Atomic absorption standards (Merck) were diluted with deionized water and used to construct a standard curve. Values were expressed as the average of three replicates from each sample expressed in mg/100 g of dry matter (DM).

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Total sugars

Total sugars in the mixture of pulp and candy were determined by the Antrona method (Dische, 1962) using a spectrophotometer (Beckman 640 B) at 620 nm. Results were expressed as the percentage (%) of total sugars.

Ascorbic acid

Determination of ascorbic acid in pulps was performed by the colorimetric method using 2,4-dinitrophenylhydrazine, according to Strohecker and Henning (1967). Analysis was performed with a spectrophotometer (Beckman 640 B), using a computerized system, and results expressed in mg of ascorbic acid (mg/100 g) of fresh pulp.

Total phenolic content

Two grams of pulp sample was extracted with 20 mL of 50% methyl alcohol. The mixture was homogenized and placed in the dark for 1 h at room temperature. After this period, the mixture was centrifuged at 14000 rpm for 15 min. The supernatant was collected and stored in flasks. The residue was dissolved in 20 mL of 70% acetone, homogenized and placed in the dark for 1 h at a temperature of 8°C. Then it was centrifuged at 14000 rpm for 15 min. The supernatant was transferred to a volumetric flask and volume adjusted to 50 mL with distilled water (Larrauri et al., 1997; Souza et al., 2012). Determination of total phenolic content was made as described by Kuskoski et al. (2005), using the Folin-Ciocalteu assay (aliquot of 0.5 mL from each sample is mixed with 2.5 ml of 10% Folin-Ciocalteu reagent). The reaction was neutralized with 2 mL of 4% sodium carbonate. Tubes were vortexed and placed in the dark for 2 h. Results were expressed in milligram gallic acid equivalents of 100 g of sample (mg GAE/100 g).

Antioxidant activity

Extracts were obtained following the same procedure used for the determination of total phenolic compounds. Determination of antioxidant activity was based on the extinction of 2,2-diphenyl-1-picryl hydrazyl (DPPH 60 µM) radical absorption, according to Rufino et al. (2009). For the determination of antioxidant activity, 0.1 mL aliquot of each extract was transferred in the dark to test tubes obtaining 3.9 mL of 60 µM DPPH and homogenized by shaking. Reading was performed at 515 nm with a spectrophotometer (Beckman 640 B), using a computerized system and monitored every minute by observing the reduction in absorbance until stabilization. The results were expressed as the percentage (%) of seizure induced free radical (% SFR), according to Equation 1:

$$\%SFR = \frac{(CA - SA)}{CA} \times 100 \quad (1)$$

CA = control absorbance; SA = sample absorbance.

Statistical analysis

Data of ascorbic acid, total phenolic compounds, antioxidant activity, and total sugars in pulp samples were evaluated and the genetic dissimilarity between each pair of individuals was determined using Euclidean distance. The Unweighted Pair Group Method Arithmetic Average (UPGMA) was used to group the genotypes based on their genetic similarity. Principal component analysis (PCA) was also performed for the chemical constituents. All analyses were carried out with Statistica Software 7 (Statsoft, 2007).

RESULTS AND DISCUSSION

The highest pH value was found in group 5 of Mirabela. Groups 3, 4, 6 and 8 had the lowest pH values compared to the other groups or locations (Table 1). Additionally, Brix values were the highest in group 2 of Mirabela (11.6° Bx). In Bonito de Minas, the largest Brix values were observed in groups 1, 4 and 8. The lowest Brix value was found in group 5 of Bonito de Minas (Table 1). In general, the highest acidity values were observed in the population of Cristália, especially in groups 1, 2 and 6. The highest ratio of soluble solids to titratable acidity was found in groups 1, 3 and 5 of Bonito de Minas, while the lowest values were obtained in group 3 of Abóboras (2.62) and Cristália (2.77) (Table 1).

Acidity values found in this study are similar to those reported by Silva et al. (2006). They observed that the pulp of *Butia eriospatha* had an average acidity of 2.20 and 1.91% in Paraná and Santa Catarina, respectively. Average Brix values for *B. capitata* ranged from 6.4 to 7.7°Bx in Santa Catarina and Paraná, and were similar to those found by Silva et al. (2006). In Groups 5 and 8 of Cristália and Bonito de Minas, Brix values were higher than those previously reported (Table 1). The pH of *B. capitata* was higher compared to *B. eriospatha*. The latter had values that ranged from 2.93 in fruits collected from Paraná to 3.06 in those from Santa Catarina (Silva et al., 2006). Results obtained from the analysis of *Acrocomia aculeata*, a palm tree in Cerrado biome, showed that pH and acidity values varied between locations. *A. aculeata* is less acidic compared to *B. capitata*; fruits collected from Presidente Epitácio and Dourados had acidity values of 69 and 0.73% and pH values of 5.7 and 6.9, respectively (Sanjinez-Argandoña and Chuba, 2011). In addition, in *Euterpe oleracea* and *Euterpe edulis,* acidity values were lower compared to *B. capitata*; acidity values were 0.19% in both species, and pH values were 4.84 in *E. oleracea* and 5.0 in *Euterpe edulis*. Brix values of *B. capitata* were higher than those of *E. edulis* and *E. oleracea* (3.03 Bx and 2.7°Bx, respectively) (Ribeiro et al., 2011).

In fruits, organic acids influence the taste, odor, color, stability, and maintenance of quality, because they are intermediate products of the respiratory metabolism. Therefore, acidity is important for determining ripeness and conservation status of the product (Oliveira et al., 1999). A comparative analysis with other palm fruits showed that *B. capitata* had a higher percentage of acidity in juice, which is the fruit's produce most used in the Northern Minas Gerais. The value of pH influences

Table 1. Values of pH, Brix, titratable acidity and soluble solids to titratable acid ratio of *Butia capitata* pulp collected from different populations and locations in Northern Minas Gerais.

Location	Groups of plants							
	G1	G2	G3	G4	G5	G6	G7	G8
pH								
Abóboras	3.39±0.00	3.36±0.10	3.34±0.00	3.41±0.00	3.46±0.00	3.42±0.00	3.36±0.00	3.52±0.00
Mirabela	3.49±0.00	3.49±0.1	3.10±0.00	3.15±0.00	4.00±0.00	3.15±0.00	3.33±0.10	3.14±0.00
Bonito de Minas	3.67±0.00	3.54±0.00	3.53±0.10	3.48±0.00	3.53±0.00	3.41±0.00	3.37±0.00	3.38±0.00
Cristália	3.31±0.00	3.36±0.00	3.36±0.00	3.38±5.40	3.26±0.00	3.22±0.00	3.41±0.00	3.58±0.00
°BRIX								
Abóboras	9.0±0.00	7.0±0.00	6.6±0.50	7.6±0.50	8.0±0.00	8.0±0.00	7.0±0.00	7.0±0.00
Mirabela	7.6±0.50	7.0±1.0	11.6±1.50	7.0±3.0	7.6±0.50	8.6±0.50	9.6±0.50	9.6±0.50
Bonito de Minas	9.6±1.50	8.0±0.00	6.6±0.50	9.0±2.00	6.0±1.00	8.6±0.50	8.0±0.00	10.0±1.0
Cristália	8.6±0.50	8.6±0.50	8.6±0.50	7.6±5.0	10.0±2.0	8.6±1.50	8.6±0.50	6.6±0.50
Titratable acidity (%)								
Abóboras	2.54±0.10	1.96±0.40	2.48±0.00	2.21±0.10	2.02±0.10	2.35±0.30	2.22±0.10	1.96±0.00
Mirabela	2.02±0.10	1.96±0.00	1.96±0.50	2.28±0.20	2.15±0.20	1.83±0.10	1.76±0.20	2.09±0.00
Bonito de Minas	1.70±0.30	1.76±0.20	1.50±0.2	1.76±0.2	1.57±0.0	1.70±0.1	1.96±0.0	1.70±0.0
Cristália	2.61±0.00	2.61±0.00	2.02±0.10	2.15±6.50	2.28±0.10	3.20±0.10	2.54±0.10	2.35±0.30
STT/ATT - ratio								
Abóboras	3.53±0.01	3.67±0.20	2.62±0.10	3.10±0.10	3.95±0.10	3.43±0.40	3.16±0.20	3.57±0.00
Mirabela	3.70±0.10	3.57±0.50	6.31±2.50	3.15±1.60	3.48±0.10	4.65±0.10	5.45±0.90	4.64±0.20
Bonito de Minas	6.58±1.0	6.29±0.70	7.40±1.0	6.29±0.70	7.02±0.00	6.50±0.50	5.61±0.00	6.48±0.00
Cristália	3.25±0.20	3.25±0.20	4.19±0.20	3.47±1.30	4.35±0.80	2.65±0.40	3.33±0.10	2.77±0.10

Mean value ± standard deviation of fruit pulp weight; n = 3; *AB, Abóboras; MI, Mirabela; BM, Bonito de Minas; CR, Cristália.

several factors such as enzyme activity, maintenance of pulp flavor and aroma, food spoilage, and growth of microorganisms in ripe fruit (Oliveira et al., 1999). The ratio of soluble solids to titratable acidity is used as an index of total sugars in fruits, indicating the degree of maturity. Soluble solids consist of water-soluble compounds, as sugars, acids, ascorbic acid, and some pectin (Oliveira et al., 1999).

The percentage of total sugars varied due to location and plant population. The highest values were found in groups 2 and 3 (3.35 and 3.22%, respectively) of Mirabela (Table 2). In Bonito de Minas, the highest value was observed in group 1. The lowest percentages of total sugars were observed in groups 3, 4, 5 and 8 of Cristália (Table 2). The values of total sugars in the pulp of *B. capitata* were up to 7 times lower than those found in *A. aculeate*, collected in Presidente Epitácio (11.58%) and Dourados (14.53%) (Sanjinez-Argandoña and Chuba, 2011). In fruits, as banana, the total sugars ranged from 19.3 to 24.4% depending on the genotype (Cerqueira et al., 2004). In genotype of *Spondia smombin,* values ranged from 6.28 to 11.58% (Pinto et al., 2003). Overall, the pulp had a relatively low amount of sugar compared to other *B. capitata* palm trees or other species.

The content of ascorbic acid was higher in group 2 of Cristália (Table 2). In Abóboras, the highest means were found in Groups 5 and 7, while in Mirabela these were observed in group 7. Groups 6 and 7 had low amounts of carbohydrate, which were 32.40 and 38.03 mg/100 g fw, respectively. According to Ramful et al. (2011), fruits can be classified into three categories with respect to the amount of ascorbic acid: low (<30 mg/100 g), medium (30-50 mg/100 g) and high (> 50 mg/100 g). In this study, there were plants in group 1 of Cristália with values higher than 50 mg/100 g, although population from Mirabela showed valued considered medium.

Ascorbic acid in *B. capitata* ranged from 38 to 73 mg/100 g fw in previous studies (Faria et al., 2008). In *B. eriospatha*, levels of this nutrient varied due to location and the values found in samples were 70.44 mg/100 g (Paraná) and 17.61 mg/100 g fw (Santa Catarina) (Silva et al., 2006).

In *A. aculeate*, ascorbic acid had an average of 11.46 mg/100 g fw in fruits obtained from Presidente Epitácio and 34.67 mg/100 g in those collected from Dourados (Sanjinez-Argandoña and Chuba, 2011). Data of ascorbic acid values from other palms or fruits as *Citrus sinensis* (62.50 mg/100 g) and *Citrus reticulate* (32.47 mg/100 g), demonstrated that *B. Capitata* is an excellent source of vitamin C.

Table 2. Total sugars, ascorbic acid, total phenolics and antioxidant activity of *B. capitata* pulps from different groups of plants and populations in Northern Minas Gerais.

Locations*	Groups of plants							
	Total sugars (%)							
	G1	G2	G3	G4	G5	G6	G7	G8
AB	2.30±0.10	2.03±0.20	0.93±0.20	1.46±0.10	1.92±0.10	0.61±0.10	1.14±0.00	1.60±0.10
MI	2.11±0.20	3.22±0.00	3.35±0.00	2.22±0.00	1.29±0.00	1.94±0.00	1.69±0.00	2.68±0.10
BM	2.84±0.20	1.86±0.20	1.48±0.10	1.60±0.20	1.68±0.20	1.78±0.10	1.56±0.10	1.72±0.60
CR	1.29±0.40	1.52±0.20	0.88±0.30	0.66±0.10	0.79±0.00	1.04±0.20	1.06±0.30	0.76±0.00
Ascorbic acid (mg/100 g)								
AB	61.80±1.2	66.68±2.1	74.77±10.4	76.12±6.8	87.93±3.0	64.95±8.0	83.24±0.9	67.04±11.8
MI	62.75±7.2	82.85±13.1	55.43±0.00	59.42±9.6	54.53±5.0	38.04±4.8	32.40±2.0	57.95±1.3
BM	71.91±5.3	62.48±9.2	62.62±13.1	63.97±2.3	52.23±3.3	70.36±5.1	64.11±4.5	62.76±7.2
CR	30.53±27.7	92.43±0.5	83.06±17.5	86.85±5.6	82.41±9.9	59.19±7.1	85.08±0.6	85.14±8.9
Total phenolic content (mg/100 g)								
AB	716.89±250.3	545.12±3.0	607.71±42.7	528.15±155.1	528.33±43.2	583.02±28.9	520.52±112.3	413.76±16.5
MI	578.51±36.8	534.24±6.2	615.20±154.1	776.13±0.7	717.45±115.5	767.16±3.2	821.66±70.9	642.86±9.1
BM	368.14±0.8	337.26±62.9	649.46±74.4	573.02±223.2	729.21±20.5	392.18±62.8	536.84±50.4	536.84±50.4
CR	571.11±8.80	401.43±21.9	360.42±18.2	735.91±36.9	369.44±26.8	531.69±42.1	420.69±15.2	649.23±2.50
Antioxidant activity (%)								
AB	35.99±11.9	40.15±12.2	35.68±4.3	33.46±2.5	40.73±3.0	27.77±0.30	28.74±0.20	33.78±0.50
MI	39.33±6.2	28.92±0.2	21.31±2.9	17.20±16.1	44.80±5.0	44.80±5.0	38.21±2.60	27.49±1.90
BM	26.21±0.10	24.96±0.5	19.57±3.30	23.08±0.20	38.95±4.7	39.86±8.0	23.73±2.2	18.64±5.50
CR	37.69±1.40	32.46±4.10	47.71±6.40	30.97±27.50	35.18±6.00	31.14±2.50	58.39±0.30	43.73±13.40

Mean value ± standard deviation of fruit pulp weight; n = 3; *AB, Abóboras; MI, Mirabela; BM, Bonito de Minas; CR, Cristália.

The highest value of phenolic compounds was observed in group 7 of Mirabela (Table 2). In Cristália, the highest average was obtained in groups 4 and 8, followed by group 1. The lowest phenolic compounds were obtained in group 3 of Cristália and the same group of Bonito de Minas (Table 2). According to Vasco et al. (2008) fruits can be classified into three different categories based on their concentration of phenols: Low (<100 mg GAE 100 g), medium (100-500 GAE mg100 g) and high (>500 GAE mg100 g). In general, the values found in some groups of Cristália were considered high, while the values in the groups of Bonito de Minas would be characterized as medium.

The total amount of phenolic compounds in the pulp of *B. capitata* was higher than this in many palm species and other plants. Biglari et al. (2008) reported amounts that ranged from 2.89 to 6.64 mg/100 g in different genotypes of *Phoenix dactylifera*; Jacques et al. (2009) reported an average of 328.6 mg/100 g in *B. odorata* and Faria et al. (2008) reported amounts that ranged from 163 to 250 mg/100 g fw in *B. capitata*. However, Shahdadi et al. (2015) observed a variation in amounts of phenols in four fruit ripening stages of *Phoenix dactylifera*, with values ranging from 2.89 to 4.82 mg/100 g fw in mature fruits. These compounds are essential to plant growth, reproduction and defense against

Table 3. Dry matter basis phosphorus, potassium, and magnesium of *B. capitata* pulp obtained from different groups of plants and populations in northern Minas Gerais.

Locations	Groups of plants							
	G1	G2	G3	G4	G5	G6	G7	G8
Phosphorus (mg/100 g)								
Abóboras	0.19±0.00	0.19±0.01	0.19±0.01	0.19±0.00	0.18±0.01	0.19±0.00	0.19±0.00	0.19±0.00
Mirabela	0.14±0.01	0.15±0.00	0.14±0.00	0.15±0.00	0.15±0.00	0.16±0.00	0.16±0.01	0.16±0.01
Bonito de Minas	0.15±0.00	0.17±0.00	0.18±0.00	0.18±0.01	0.17±0.00	0.18±0.00	0.18±0.00	0.18±0.00
Cristália	0.14±0.00	0.13±0.00	0.16±0.00	0.13±0.00	0.14±0.00	0.17±0.00	0.16±0.00	0.14±0.00
Potassium (mg/100 g)								
Abóboras	1.22±0.04	1.18±0.01	1.25±0.03	1.50±0.02	1.99±0.00	1.44±0.01	1.07±0.02	1.09±0.01
Mirabela	1.21±0.01	1.32±0.05	1.30±0.1	1.33±0.00	1.35±0.00	1.50±0.02	1.16±0.00	1.34±0.00
Bonito de Minas	1.34±0.00	1.42±0.00	1.20±0.01	1.23±0.00	1.48±0.00	1.34±0.03	1.53±0.00	1.22±0.00
Cristália	1.40±0.05	1.58±0.01	1.15±0.00	1.52±0.00	1.39±0.01	1.53±0.04	1.67±0.00	1.19±0.01
Magnesium (mg/100 g)								
Abóboras	0.09±0.00	0.07±0.00	0.08±0.01	0.08±0.01	0.06±0.01	0.07±0.00	0.07±0.00	0.07±0.01
Mirabela	0.04±0.00	0.04±0.00	0.06±0.00	0.05±0.01	0.08±0.00	0.05±0.02	0.05±0.00	0.05±0.00
Bonito de Minas	0.06±0.00	0.05±0.00	0.07±0.00	0.05±0.00	0.07±0.00	0.06±0.00	0.06±0.00	0.05±0.00
Cristália	0.08±0.00	0.07±0.00	0.09±0.00	0.08±0.00	0.07±0.00	0.10±0.02	0.10±0.02	0.08±0.00

Mean value ± standard deviation of fruit pulp weight; n = 3. *AB, Abóboras; MI, Mirabela; BM, Bonito de Minas; CR, Cristália.

pathogens. Phenols have high antioxidant activity due to their ability to sequester free radicals and donate electrons and hydrogens (Balasundram et al., 2006). The results demonstrated that *B. capitata* presents higher antioxidant activity than other palm trees, given the high values found in this study (>500 GAE mg100 g).

Ascorbic acid and phenolic compounds in plants are mainly influenced by environmental conditions, as light and temperature, genetic variation, soil type, and stress on maturation stage (Lee and Kader, 2000). For example, in tomato, the values of ascorbic acid, total phenols, and antioxidant capacity relate positively to increased light and temperature (Raffo et al., 2006). However, genetic factors seemed to influence these parameters in tomato as reported by Caliman et al. (2010). More specifically, ascorbic acid values were higher for variety 'Santa Clara' (17.71 mg/100 g), compared to'BGH-320' and 'Carmen', which had an average of 13.00 mg/100 g, under the same cultivation conditions. The highest average of antioxidant activity was obtained in group 7 of Cristália (58.39%). The lowest antioxidant activity was observed in group 4 (17. 20%) of Mirabela (Table 2). Antioxidant activity of *B. capitata* was similar to values obtained in other palm species, as in *E. edulis* (41.73%) (Lima et al., 2012) and higher than *Citrus reticulate* (29.30%) and *Citrus sinensis* × *Citrus reticulata* (12.78%) (Couto and Canniatti-Brazaca, 2010).

Averages of phosphorus concentration, in samples obtained from Abóboras, were higher compared to other regions; however, the amount of this mineral was similar between the groups, except of 5 of them (Table 3). The lowest values of phosphorus were observed in groups 2 and 4 of Cristália and group 1 of Mirabela (Table 3). Potassium concentration was the highest in some samples obtained from Abóboras (1.99%) and seven groups of Cristália (1.67%). The lowest amounts of potassium were obtained in group 7 of Abóboras and the same group of Mirabela. Magnesium values were the highest in group 1, followed by groups 3 and 4 of Abóboras. The values of magnesium in group 6 and 7 of Cristália were higher, compared to the same groups from other locations (Table 3).

The highest averages of copper were obtained in groups 4, 5 and 6 of Abóboras followed by Group 2 of Cristália. The lowest values were observed in groups 5 and 6 of Bonito de Minas (Table 4). Manganese values in group 4 of Abóboras and Group 5 and 6 of Cristália were higher than the minor amounts found in groups 1 and 2 of Mirabela. Zinc was abundant in all groups. The highest averages of zinc were obtained in group 5, 6 and 7 of Cristália (Table 4). Iron was found in great amounts in *B. capitata*. The highest averages were observed in group 5 of Bonito de Minas, Group 4 of Abóboras and group 6 of Mirabela, while the lowest averages were obtained in group 2 of Mirabela and group 8 of Bonito de Minas (Table 4).

B. capitata has higher potassium and iron amounts than their fruits. Banana is rich in potassium and its content ranges from 297 to 341 mg/100 g fw (Ramos et al., 2009). Generally, the pulp of palm trees is a good source of potassium; reported values ranged from 77.19 and 125.08 mg/100 g in *Euterpe precatoria* (Yuyama et al., 2011), and were in average 766 mg $100g^{-1}$ in *A. aculeate* (Ramos et al., 2008). Iron is present in relatively

Table 4. Dry matter basis copper, manganese, zinc, and iron of *B. capitata* pulp obtained from different groups of plants and populations in Northern Minas Gerais.

Locations*	Groups of plants							
	G1	G2	G3	G4	G5	G6	G7	G8
Copper (mg/100 g)								
AB	0.50±0.01	0.44±0.01	0.32±0.01	0.41±0.00	0.67±0.01	0.54±0.01	0.39±0.05	0.45±0.01
MI	0.41±0.01	0.41±0.02	0.46±0.02	0.40±0.00	0.41±0.03	0.36±0.02	0.40±0.03	0.48±0.00
BM	0.31±0.00	0.34±0.01	0.34±0.00	0.36±0.04	0.29±0.00	0.28±0.01	0.39±0.02	0.35±0.00
CR	0.59±0.03	0.62±0.03	0.55±0.00	0.66±0.01	0.50±0.00	0.64±0.02	0.52±0.00	0.51±0.00
Manganese (mg/100 g)								
AB	0.63±0.00	0.45±0.00	0.83±0.02	2.41±0.01	0.54±0.01	1.09±0.01	0.60±0.06	0.51±0.02
MI	0.38±0.01	0.36±0.03	0.96±0.01	0.89±0.01	1.41±0.03	1.32±0.01	0.90±0.46	0.65±0.00
BM	1.71±0.00	0.90±0.00	1.17±0.02	1.13±0.01	3.12±0.02	1.07±0.00	1.23±0.02	0.87±0.00
CR	1.03±0.00	1.03±0.00	1.58±0.01	1.82±0.02	1.28±0.01	2.20±0.06	1.44±0.01	1.10±0.52
Zinc (mg/100 g)								
AB	0.98±0.01	0.92±0.00	0.86±0.00	0.87±0.01	0.97±0.02	0.97±0.03	0.94±0.01	0.99±0.03
MI	0.80±0.23	0.77±0.01	0.81±0.01	0.91±0.01	1.05±0.02	0.81±0.05	0.78±0.01	0.90±0.00
BM	0.83±0.00	0.80±0.01	0.78±0.00	0.72±0.01	0.95±0.00	0.63±0.00	0.87±0.00	0.76±0.05
CR	0.95±0.01	1.00±0.01	1.17±0.00	1.23±0.00	1.23±0.02	1.28±0.00	1.34±0.00	0.94±0.00
Iron (mg/100 g)								
AB	5.04±0.01	4.06±0.01	3.52±0.01	58.53±0.01	7.92±0.01	3.10±0.00	3.74±0.01	3.52±0.01
MI	3.20±0.00	2.60±0.06	6.25±0.00	17.54±0.00	26.71±0.01	45.72±0.00	3.72±0.01	7.02±0.01
BM	9.79±0.01	3.04±0.01	4.26±0.00	6.41±0.00	83.94±0.02	3.82±0.00	4.45±0.00	2.63±0.00
CR	5.73±0.02	4.35±0.00	3.48±0.00	3.28±0.00	3.92±0.01	26.02±0.00	3.37±0.00	17.40±0.12

Mean value ± standard deviation of fruit pulp weight; n = 3; *AB, Abóboras; MI, Mirabela; BM, Bonito de Minas; CR, Cristália.

larger amounts than their microelements in *E. edulis* (46.6 mg/100 g) (Ribeiro et al. 2011) and *A. aculeate* (7.71mg/100 g) (Ramos et al., 2008). Zinc amount was relatively higher in *E. precatoria*, ranging from 163.43 to 318.32 mg/100 g, compared to other plant species (Yuyama et al., 2011).

Palm fruit is a popular food in the northern region of Minas and is offered to children at schools and daycare centers. *B. capitata* pulp has high nutritional value since it is rich in ascorbic acid, phenolic compounds, antioxidants and fiber. Results obtained in this study may be used for an initial nutritional characterization and the development of product labels. They may also assist to future breeding, conservation, and educational programs.

UPGMA allowed the identification of five major groups (Figure 1). Group A included groups of plants from all evaluated populations, most of them obtained from Mirabela. It is noteworthy that group A included plants highly similar in ascorbic acid, sugars, total phenols and antioxidant activity. Group B had three sub-groups, including samples of population from Cristália and Abóboras. Group C included the highest plants from all populations. Group D and E included groups of populations with low similarity in ascorbic acid, sugars, total phenols and antioxidant activity.

In principal components analysis (PCA), the first axis represented 42.15% of total variation and the second axis represented 29.90% of total variation. In Figure 2, the distance of Cristália groups 7 and 3 from other groups was highlighted. Most individuals were similar in the middle of the dendogram. UPGMA and PCA were also effective for separating and grouping 18 different fruit species for ascorbic acid, total phenols, carotenoids, and flavonoids. Barreto et al. (2009) supported that this analysis could differentiate related species and those with low similarity for the production of bioactive compounds. In *A. carambola*, PCA allowed the grouping of pulps collected at harvest time and analyzed for various bioactive compounds (Zainudin et al., 2014). The separation of plants into different groups can help breeders to select distinct *B. capitata* genotypes, and can guide producers for the best harvest time of fruit. Fruits are manually harvested by native populations and sold at local markets or the extracted pulps are supplied by cooperatives to school lunch programs.

Conclusion

The results demonstrated that *B. capitata* pulp is rich in

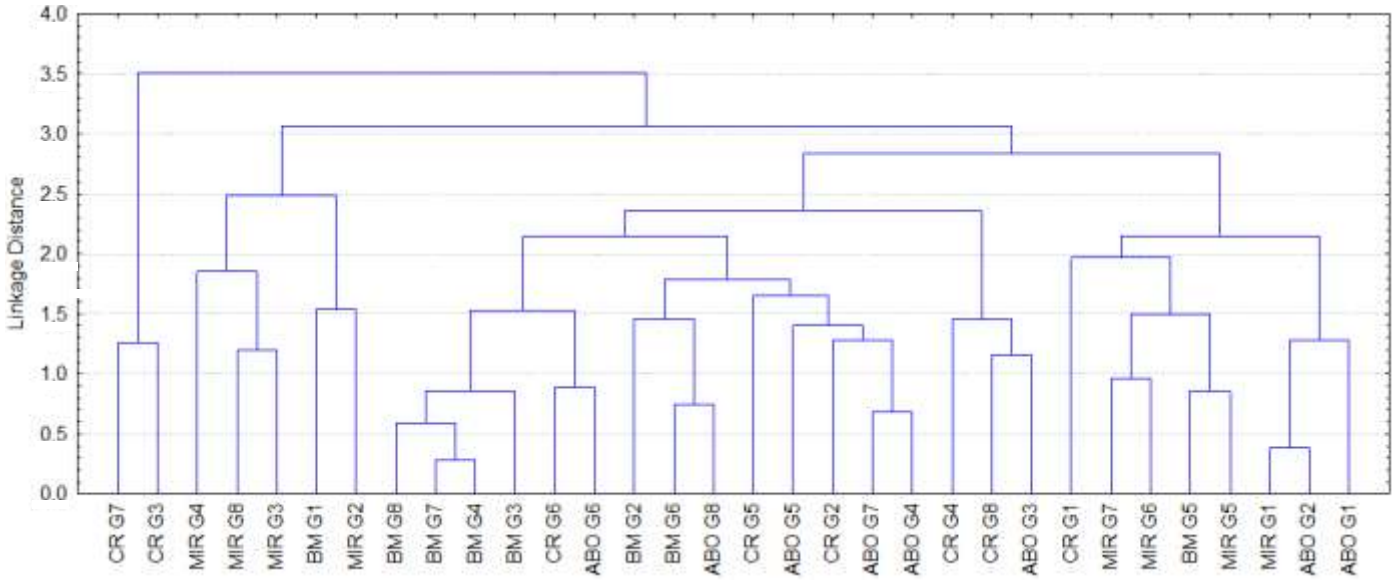

Figure 1. UPGMA dendrogram showing genetic similarity between *B. capitata* populations collected from Bonito de Minas (B), Mirabela (M), Abóboras (A), Cristália (C) in Northern Minas Gerais.

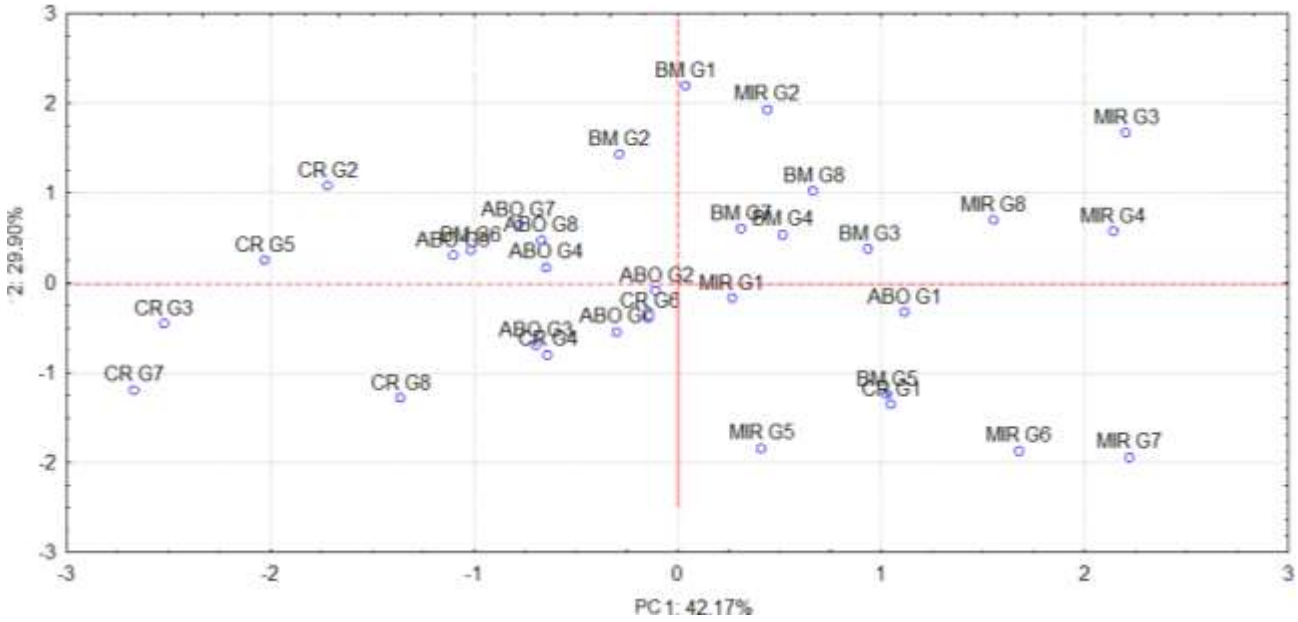

Figure 2. Principal components analysis (PCA) of ascorbic acid, phenolic compounds, total sugars, and antioxidant activity among different populations and groups of *B. capitata* collected from Bonito de Minas, Mirabela, Abóboras and Cristália in Northern Minas Gerais.

ascorbic acid (with average values higher than 65 mg/100 g fw) and minerals, especially potassium and iron. It contains large amounts of phenolic compounds and has a good antioxidant capacity. This composition varies due to soil composition, climatic conditions, and genotype. Multivariate analysis grouped and separated populations that were genetically similar or dissimilar for ascorbic acid, antioxidant activity, total phenolic compounds and total sugars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Synthesis of silver nanoparticles using wild *Cucumis anguria*: Characterization and antibacterial activity

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In the present study silver nanoparticles were synthesized using wild *Cucumis anguria* leaf extract using the green route which is a simple, cost-effective and an environmentally friendly technique. When the aqueous silver ions were exposed to the wild *C. anguria* leaf extract, they were reduced and this resulted in the formation of silver nanoparticles. The conditions of synthesis were optimized by adjusting the pH, time and amount of leaf extract. The synthesized silver nanoparticles were characterized by different techniques, namely ultra-violet visible spectroscopy, Fourier Transform Infrared Spectroscopy, X-ray diffraction and transmission electron microscopy. The ultra violet-visible spectrum of the synthesized silver nanoparticles shows a maximum peak at 420 nm. Fourier transform infrared spectroscopy results show the presence of alcohols, aromatics and amines suggesting the presence and binding of proteins with silver nanoparticles. Transmission electron microscopy analysis shows that the silver nanoparticles synthesized were spherical in shape with their sizes ranging between 11 and 27 nm depending on the pH conditions. X-ray diffraction analysis results show the presence of silver metal nanoparticles and their crystalline nature. The antibacterial activity of the silver nanoparticles synthesized was evaluated against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacterial and kanamycin was used as a control. The green chemistry route has proven that silver nanoparticles can be synthesized using *C. anguria* leaf extract in which biomolecules effectively act as reducing and capping agents.

Key words: Silver nanoparticles, green synthesis, *Cucumis anguria,* antibacterial activity.

INTRODUCTION

Nanotechnology is an important scientific field which deals with the production, manipulation and use of materials in the nano range. It deals with all the processes that take place on the nanometer scale that is from approximately 1 to 100 nm (Duncan, 2011; Ahmed et al., 2016). The synthesis of nanoparticles has recently gained interest due to their wide variety of applications in areas such as medicine, catalysis, electronics, photonics, computer transistors, sensing, antimicrobial activity, anti-inflamatory and so on (Jeong et al., 2005; Moon et al.,

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Figure 1. Leaves of wild *Cucumis anguria*.

2005; Lee and El-Sayed, 2006; Gong et al., 2007; Alexander, 2009; Chen et al., 2009; Gopinath et al., 2012; Mukherjee et al., 2002). Most of these desirable characteristics of the nanoparticles are attributed to their extremely small size and large surface volume compared to their bulk counterparts. Nanoparticles possess size and shape (cylinders, rods, spheres etc) dependent properties which are of interest for such applications (Narayanan, 2012; Roy and Das, 2015). Silver and gold nanoparticles have been particularly used with promising results as bactericides and anticancer agents (Xu et al., 2006; Devara et al., 2013).

Nanomaterials can be made of different materials and the common materials include silicates, metal oxides, non-oxide organics, ceramic polymers, metals, carbon and bio-molecules (Nagarajan, 2008). Basically there are two main groups into which nanoparticles can be classified, that is, organic and inorganic.

Various methods have been used for synthesizing silver nanoparticles and these methods include the chemical, physical and biological methods (Chandran et al., 2006; Jha et al., 2009; Raghunandan et al., 2010; Iravani, 2011; Jacob et al., 2011; Zayed et al., 2012; Kumarasamyraja and Jeganathan, 2013; Suman et al., 2013; Rodriguez-Leon et al., 2013; Roy et al., 2015). Most chemical methods used for synthesizing nanoparticles are too expensive and time consuming and at the same time the intense use of solvents and synthetic reactants is toxic and hazardous to the environment. This has led to the green methods of nanomaterial preparation which involve a single step and involve the use of environmentally friendly reactants. Silver nanoparticles obtained by the green synthesis methods provide good candidates to be used in biological systems. The green synthesis route requires two elements for nanoparticle growth: a silver salt and a reducing agent.

It has been revealed that some bacterial strains have developed resistance against common drugs (Singh et al., 2014). This resistance to medication by disease causing organisms has caused a stir in the medicine industry and this has led to the need to develop new bactericides and virucides. Silver and silver nanoparticles have an antiseptic and disinfectant history and are able to interact with disulphide bonds of the glycoprotein/protein contents of microorganisms (Ahmed et al., 2016). The resistance to medication by pathogens has become a stern concern in public health (Singh et al. 2014), hence the need for synthesis of silver nanoparticles. The synthesized nanoparticles should be more compatible for medical applications hence the use of edible plant extracts. Therefore, the exploitation of plant extracts has materialized as a novel method for the synthesis of silver nanoparticles (Shahverdi et al., 2007). In this work *Cucumis anguria* (Gherkin), a medicinal vegetable crop was selected which is abundantly found in Zimbabwe, and has a great potential for commercial reproduction for sustainable green synthesis of AgNPs. Figure 1 shows the leaves from *C. anguria*.

Analysis of the phytochemicals found in wild *C. anguria* leaves revealed that the leaves contain very useful compounds such as flavonoids, tannins, alkaloids, saponins and steroids which possess high levels of antioxidant activity (Dzomba and Mupa, 2012). Anthraquinones and saponins present in wild *C. anguria* exhibit antibacterial and antifungal activity against most clinical pathogens. However the leaves of wild *C. anguria* have not been reported for the synthesis of silver nanoparticles. In view of this background this plant is chosen as a reducing and stabilizing agent for the green synthesis of silver nanoparticles as well as their characterization and application in therapeutics.

MATERIALS AND METHODS

Preparation of the leaf extract

The leaves of wild *C. anguria* were collected from the local area of Chiweshe, Zimbabwe (from ARDA and Visa farms) and were thoroughly washed 4 to 5 times using distilled water to remove any dust particles and any other contaminating particles. About 30 g of fresh leaves were finely chopped and placed in a heating container and about 90 ml of reagent grade water was added. The mixture was boiled for 1 h while stirring continuously. After boiling, the mixture was cooled and filtered using a vacuum pump with a Whatman paper number 1 and the filtrate was collected.

Synthesis of silver nanoparticles

About 1 mM of aqueous solution of silver nitrate was prepared and used for the synthesis of silver nanoparticles. 10 ml of the wild *C. anguria* leaf extract was treated with 90 ml of 1 mM silver nitrate solution for bio-reduction process at room temperature under static conditions. The resulting solution was incubated in the dark (to minimize the photo activation of silver nitrate). The process of synthesizing silver nanoparticles was repeated at different pH (4, 7,

9 and 11) and also at different concentrations of leaf extract by reacting different amounts (1, 2, 3 and 4 ml) of the *C. anguria* leaf extract with 10 ml of 1 mM $AgNO_3$ solution. The silver nanoparticles formed were centrifuged at 2000 rpm for 10 min and the supernatant was decanted. The resulting suspension was re-dispersed in 10 ml sterile distilled water and the centrifugation process was repeated for three times to purify the synthesized silver nanoparticles.

Characterizing the synthesized silver nanoparticles

Ultra violet-visible analysis

The biosynthesis of silver nanoparticles was monitored using a GENESYS 10S (Thermo Fisher Scientific, UK) UV-Vis spectrophotometer at the wave length range of 200 to 800 nm at different times of synthesis (after 1, 12, 24 and 48 h).The scanning was repeated using silver nanoparticles synthesized at different pH (4, 7, 9 and 11) and silver nanoparticles synthesized at different leaf extract concentrations (1l, 2, 3 and 4 ml). Distilled water was used as a blank.

Fourier transform infrared analysis

FTIR analysis was carried out to identify the various functional groups in biomolecules responsible for the bio-reduction of Ag^+ ions and capping/stabilization of silver nanoparticles. The analysis was done using a NIOLET iS5 (Fisher Thermo Scientific, UK) FTIR spectrometer. About 20 ml of wild *C. anguria* leaf extract and 20 ml of the synthesized silver nanoparticles were air dried at room temperature. The dried powder samples of the leaf extract and silver nanoparticles were analyzed in the range of 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Transmission electron microscopy analysis

The Joel JEM-2100 (Akishima-Shi, Japan) transition electron microscope was used to determine the size and shape of the synthesized silver nanoparticles. Sample preparation was done by dissolving about 10 mg of silver nanoparticles in ethanol to form a suspension. The suspension was ultrasonicated for formation of a uniform suspension of silver nanoparticles in ethanol. The suspension was then centrifuged and the supernatant was collected. A very low concentration of the supernatant was dropped on the TEM carbon coated copper grid mesh and it was allowed to dry. The dry silver nanoparticles on a carbon coated copper grid mesh were then screened and images were collected.

X-ray diffraction analysis

Sample preparation was done by grinding the silver nanoparticles into a fine powder and placing it on a sample holder. Screening was done using X'PERT-PROGoniometer (PANalytical, Japan) operating at a voltage of 40 kv and current of 40 mA with a Cu kα radiation. The scanning mode used was continuous with a scanning range (2θ) from around 4° to approximately 90°. The images obtained were compared with the Joint Committee on Powder Diffraction Standards (JCPDS) library to account for the crystalline structure.

Evaluation of antibacterial activity of the synthesized silver nanoparticles

The disc diffusion method was used in the procedure for evaluation of the antibacterial activity of the synthesized silver nanoparticles (Malabadi et al., 2005). Antibacterial activity of *C. anguria* silver nanoparticles was evaluated against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) pathogenic microorganisms. In brief, Mueller Hinton (MH) agar base plates were prepared using sterile 90 mm Petri dishes. MH agar was inoculated with MH broth culture of each bacterial species and poured over the base plates to form a homogenous layer. Filter paper discs (5 mm in diameter) were sterilized and the sterile paper discs were dipped in silver nanoparticle solution (10 µg/ml); some were placed in silver nitrate solution and some sterile discs were dipped in *C. anguria* leaf extract. These discs were then air dried under sterile conditions. The dry sterile discs were then placed onto the seeded top layer of the MH agar plates and were left for 30 min at room temperature for compound diffusion. Kanamycin was used as positive control. Plates were incubated for 24 h at 37°C and the zones of inhibition were measured and recorded in millimeters.

RESULTS AND DISCUSSION

Characterization of the synthesized silver nanoparticles

Visual observation

Visual observation was used to confirm the formation of silver nanoparticles. The yellow mixture of silver nitrate and *C. anguria* turned to a dark brown colour indicating the formation of silver nanoparticles. The colour changes in the formation of silver nanoparticles are shown in Figure 2.

This characteristic brown color of silver nanoparticles provided simple spectroscopic signature to indicate the formation of nanoparticles (Prasad et al., 2011). The formation of silver nanoparticles occurred from a few minutes to hours agreeing with reports for other plant extracts (Chanda, 2014). It was noted through visual observation that silver nanoparticle formation was rapid (as was shown by a rapid colour change) under neutral and basic conditions and this could be due to the ionization of the phenolic group present in the *C. anguria* leaf extract (Martinez-Castanon et al., 2008). The rate was noted to be slow under acidic conditions, and this can be attributed to the electrostatic repulsion of the anions present in solution (Sun et al., 2014). At basic pH there is a possibility of Ag^+ ions precipitating as AgOH.

Ultra violet-visible analysis

UV-Vis spectroscopic curves presented in Figures 2 to 4 show peaks around 425 nm. This confirms the formation of silver nanoparticles (Shankar et al., 2004). The absorption spectrum noted around 425 nm could have originated from the strong Surface Plasmon Resonance (SPR) that is due to resonant absorption of photons by silver nanoparticles (Kokila et al., 2015). Figure 3 shows that the intensity of absorption increases with time indicating the enhancement of silver nanoparticle formation with time as a result of reduction of Ag^+ ions

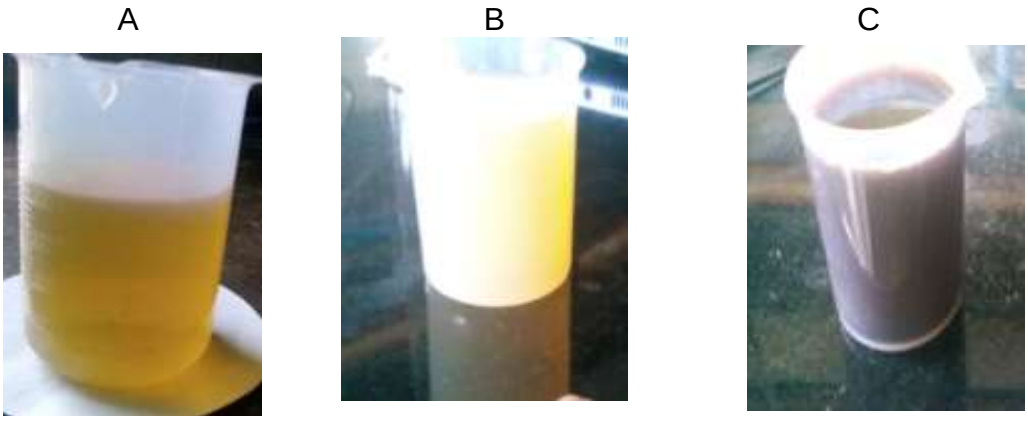

Figure 2. Colour change upon silver nanoparticle formation. (A) The colour of the leaf extract. (B) The colour of the reaction mixture at the beginning. (C) The color of the solution after 48 h of reaction.

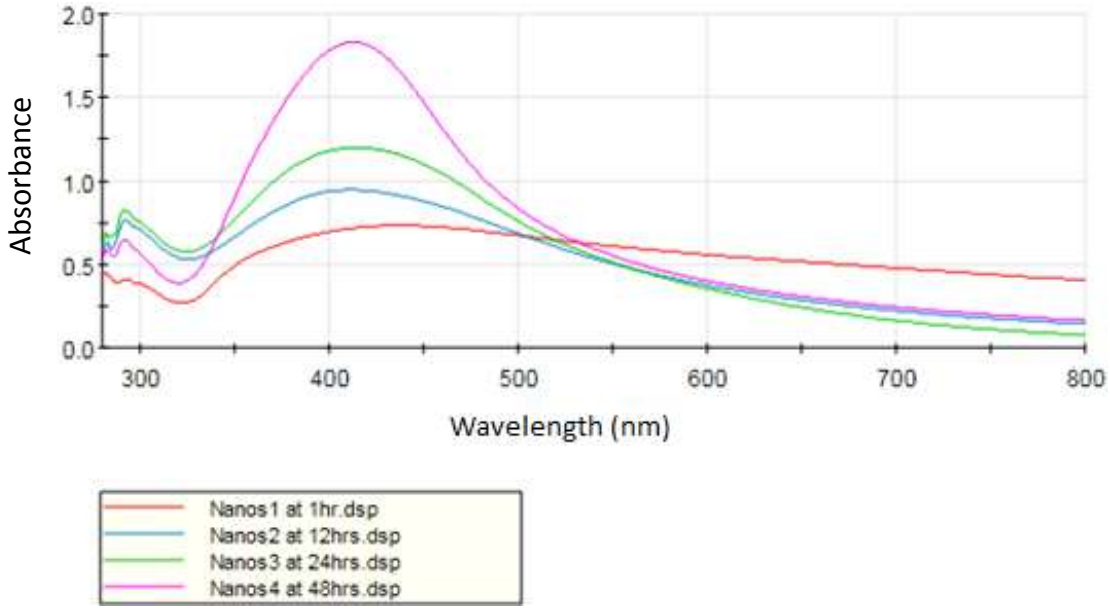

Figure 3. Absorption spectra of AgNPs (synthesized from 1 mM $AgNO_3$ and *Cucumis anguria* leaf extract) observed at four different times. The figure shows an increase of absorption intensity as a function of reaction time.

with the help of reducing agents present in the *C. anguria* leaf extract. *C. anguria* leaf extract possess natural reducing agents like flavonoids and terpenoids and saponins which are responsible for the reduction of silver ions to silver nanoparticles. The intensity of the SPR band increased without any shift in the peak wavelength with passage of time. This can be attributed to minimum polydispersion of the synthesized nanoparticles. Similar results were obtained when silver nanoparticles were synthesized from *Lippia javanica* (Kumar et al., 2015) implying that stabilizing molecules or functional groups from the two plants could be the same, or the size and shape of the synthesized nanoparticles at the specified conditions were the same.

UV-Vis analysis was also done for the silver nanoparticles synthesized at different pH and the nanoparticles at different pH were given maximum formation time before scanning them under UV-vis. The results are shown in Figure 3.

The results in Figure 4 show that fewer nanoparticles were formed under acidic conditions and the number of silver nanoparticles increased with an increase in pH as shown by an increase in intensity as the pH increased. The maximum formation of silver nanoparticles occurred

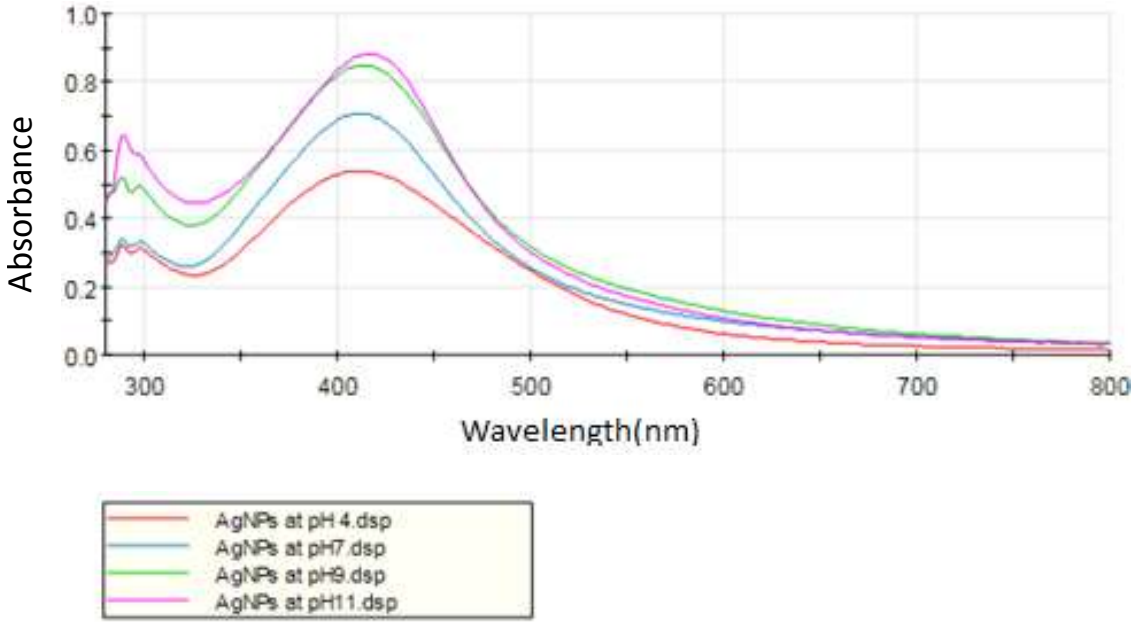

Figure 4. Absorption spectra of AgNPS (synthesized from 1 mM $AgNO_3$ and *C. anguria* leaf extract) at different pH values (pH = 4, 7, 9 and 11).

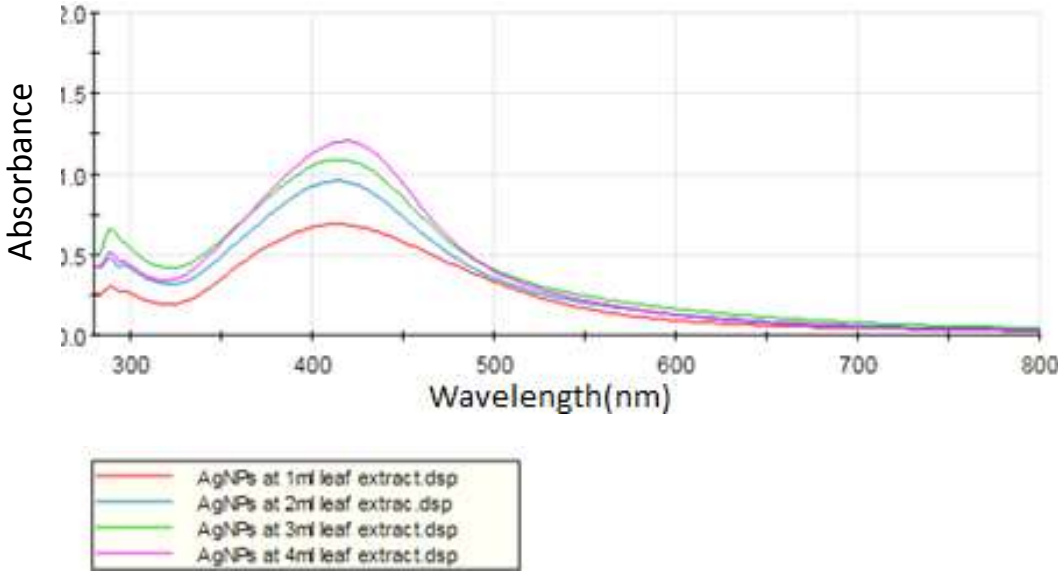

Figure 5. Absorption spectra of AgNPs of different amounts of *C. anguria* leaf extract (1, 2, 3 and 4 ml) with 10 of 1 m $MAgNO_3$ solution. The figure shows an increase of absorption intensity with an increase in the amount of leaf extract.

at pH 11. Higher SPR intensity at alkaline conditions depicts increased number of smaller silver nanoparticles (Kokila et al., 2015). Alkaline conditions favour the formation of silver nanoparticles because hydroxides get deposited on the silver nanoparticles (Oza et al., 2013). At alkaline pH, both reducing and the capping agents are efficiently reducing the silver. Also, they promote encapping of nanoparticles making them thermodynamically favorable. Figure 5 shows that an increase in the concentration of *C. anguria* leaf extract leads to an increase in the peak intensity indicating an enhancement in the production of silver nanoparticles as the concentration of leaf extract increases.

Similar results were obtained when silver nanoparticles

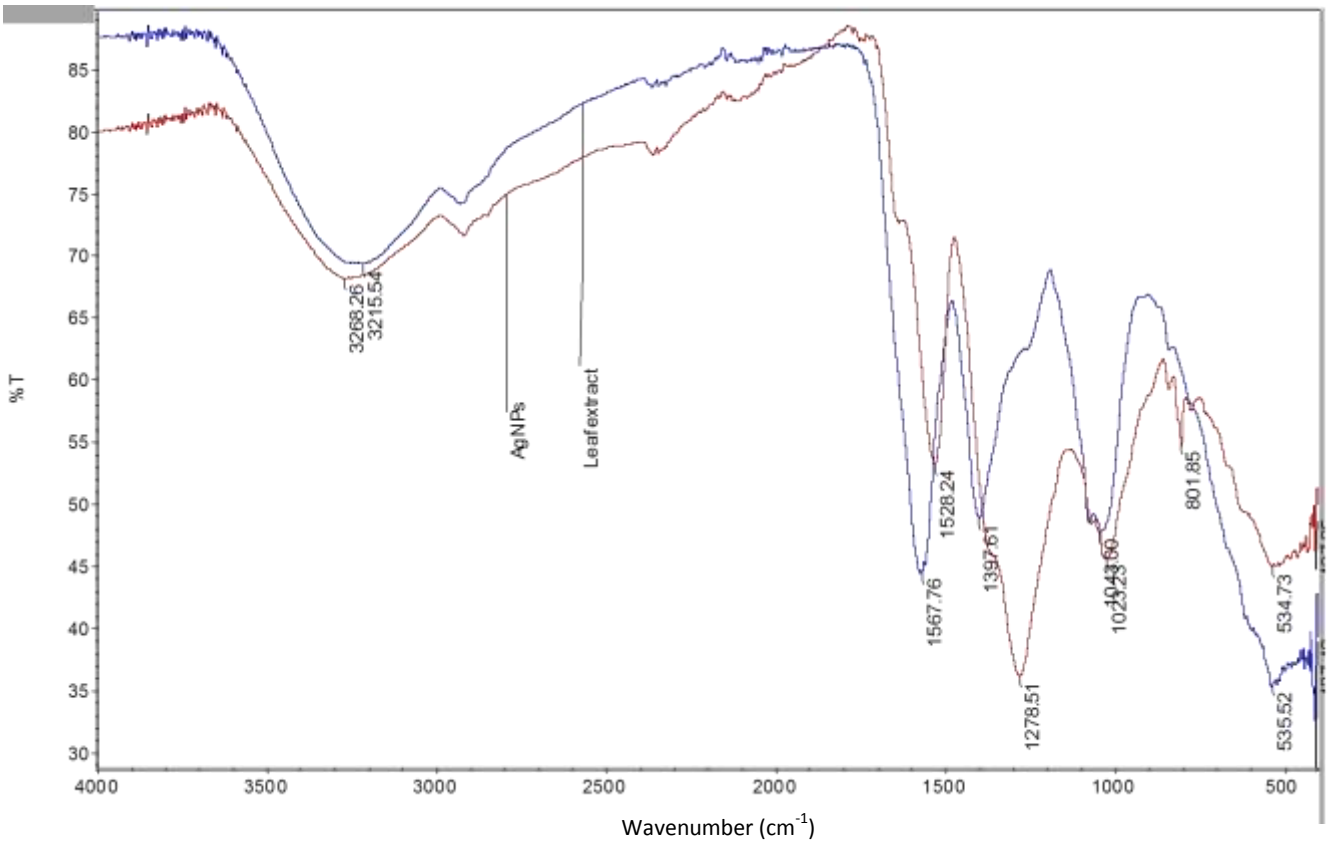

Figure 6. Combined FTIR spectra of AgNPs and *C. anguria* leaf extract.

were synthesized using *Ficus Benghalensis* leaf extract (Saware et al., 2014). All UV-Vis results show that the silver nanoparticles synthesized are spherical in shape. According to Mies theory (He et al., 2002; Kokila et al., 2015), only a single SRP band is expected in the absorption spectra of spherical metal nanoparticles whereas anisotropic nanoparticles give rise to two or more SPR bands depending on the shape of the nanoparticles. In the present study a single SPR peak was formed which suggest that the nanoparticles synthesized were spherical in shape. The broadened peaks shown by the UV-Vis results indicate that the particles were poly-dispersed (Maria et al., 2015).

Fourier transform infrared analysis

FTIR analysis was done for both the dried silver nano particles and the dried *C. anguria* leaf extract. The FTIR spectroscopic studies were performed to investigate interaction between the surface of silver nanoparticles and possible organic functional groups of constituent compounds present in the leaf extract. The band intensities in the different regions of the spectrum of *C. anguria* leaf extract and silver nanoparticles are shown in Figure 6.

The spectrum of the leaf extract shows bands at 3215.54, 1567.76, 1397.61, 1043.23, 801.20, 535.52 and 407.30 cm^{-1}. The broad and intense absorption spectrum at around 3215.54 corresponds to the O-H stretching vibrations of phenols and carboxylic acids. The shift from 3268.26 (leaf extract) to 3215.54 cm^{-1} (silver nanoparticles) may indicate the involvement of the O-H functional group in the synthesis of the silver nanoparticles (Jancy and Inbathamizh, 2012). The FTIR spectrum showed a band at 1567.76 (leaf extract) and 1528.24 cm^{-1} (silver nanoparticles) which corresponds to the bending vibration of the amide bands of the proteins (Navin et al., 2010). The peak at 1397.61 cm^{-1} (leaf extract) shows the C-N stretching vibrations of the aromatic amines and the shift of this band to 1278.51 cm^{-1} (silver nanoparticles) may predict the involvement of the aromatic amines in the synthesis of silver nanoparticles (Baishya and Kalita, 2013). The spectrum also shows a peak at 1043.23 cm^{-1} (leaf extract) which corresponds to aliphatic amines and the shift of this peak to 1023.90 (silver nanoparticles) shows the involvement of amines in the formation of silver nanoparticles (Navin et al., 2010). The peaks at 535.52 and 407.30 cm^{-1} corresponds to alkyl halide and hydroxyl groups, respectively (Preetha et

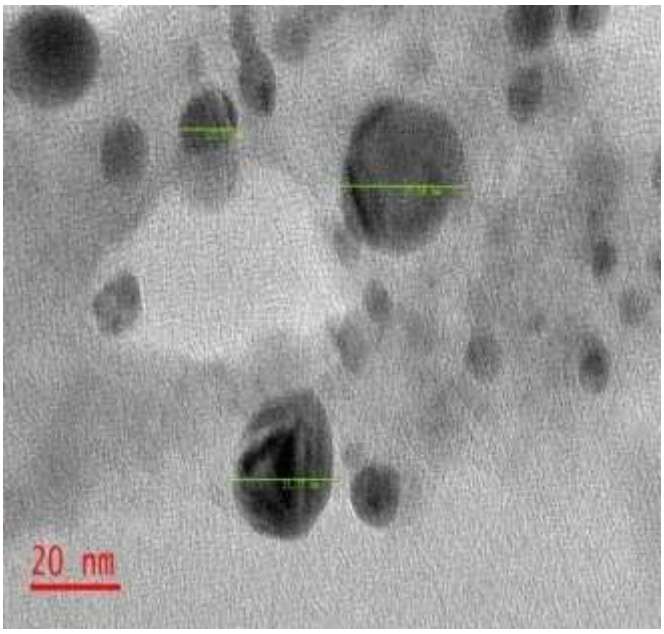

Figure 7. TEM images of silver nanoparticles at pH 4 scanned at 20 nm.

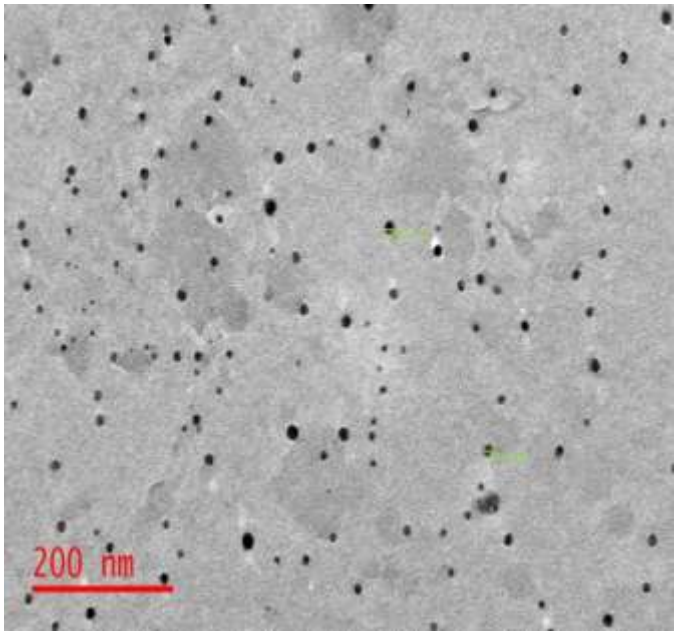

Figure 8. TEM images of silver nanoparticles at pH 7 scanned at 200 nm.

al., 2014). The shifting of the peak from 407.40 cm^{-1} (leaf extract) to 407.05 cm^{-1} (silver nanoparticles) indicates the bonding between the silver nanoparticles and the oxygen on the hydroxyl groups (Kokila et al., 2015). The shift of band position in the spectra of silver nanoparticles as compared to the spectra of the leaf extract suggest that

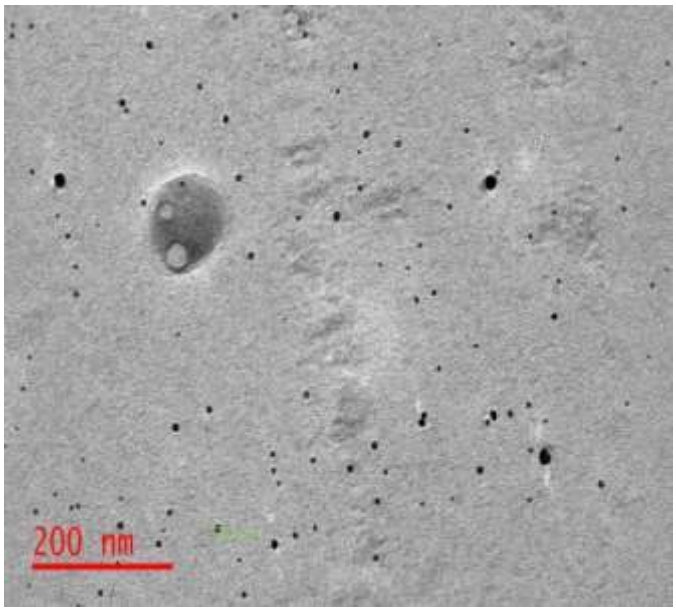

Figure 9. TEM images of silver nanoparticles at pH 11 scanned at 200 nm.

the above mentioned functional groups maybe involved in the reduction of silver ions as well as the stabilization of the nanoparticles. Studies have shown that the *C. anguria* leaf extract contains phenolic compounds, flavonoids, terpenoids, saponins and some other proteins which can cause the reduction of metal ions and the stabilization of the synthesized silver nanoparticles (Shankar et al., 2004; Chandran et al., 2006).

Transmission electron microscopy analysis

The TEM images of the silver nanoparticles synthesized at pH 4, 7 and 11 are shown in Figures 7 to 9, respectively.

The average size of the silver nanoparticles at pH 4 is around 20.64 nm, the average size of nanoparticles at pH 7 is around 13.51 nm and the average size for nanoparticles at pH 11 is around 11.01 nm. Lower pH values for example pH 4 promote the nucleation of the silver nanoparticles leading to the formation of larger nanoparticles (Kokila et al., 2015). High pH values cause electrostatic repulsions among the nanoparticles thereby leading to the formation of smaller and highly dispersed nanoparticles (Sathishkumar et al., 2009). TEM results also show that the nanoparticles synthesized are spherical in nature and this agrees well with the UV-Vis results which produced a single peak depicting the formation of spherical nanoparticles according to Mies theory (Kokila et al., 2015). Similar results were obtained when silver nanoparticles were synthesized using *C. sativus* fruit extract (Roy et al., 2015).

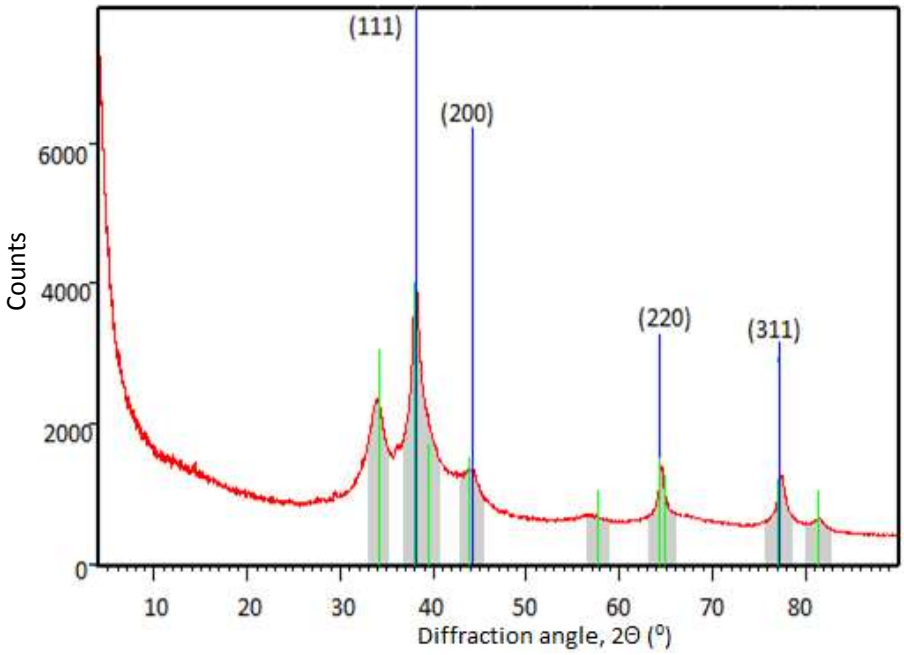

Figure 10. XRD diffractogram showing the intensity of peaks as a function of diffraction angles.

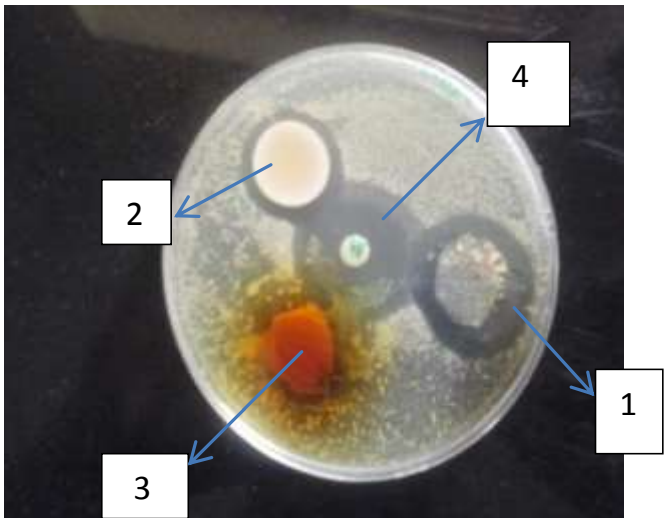

Figure 11. The antibacterial activity of (1) AgNPs, (2) $AgNO_3$, (3) leaf extract and (4) Kanamycin against *S. aureus*.

X-ray diffraction analysis

XRD results are shown on Figure 10 with the main peaks at 38.1286, 44.1242, 64.5511 and 77.4726° corresponding to the 111, 200, 220 and 311 planes, respectively. This indicatesthat the silver nanoparticles are spherical and crystalline in nature, Ibrahim (2015) with face centered cubic structure (FCC) according to data comparison with the 'data base of joint committee on Powder Diffraction Standards (JCPDS) file No. 04-0783'. Apart from these peaks which arise due to silver nanoparticles, the recorded XRD diffractogram has additional peaks at 33.9693 and 56.8604 and 81.46. This might be due to the formation of the crystalline bio-organic compounds\metallo-proteins that are present in the *C. anguria* leaf extract (Anandalakshmi et al., 2016). The peak corresponding to the 111 plane is more intense than the other planes and this suggests that the 111 plane is the most predominant orientation.

Antibacterial activity of silver nanoparticles

The antibacterial activity of the synthesized silver nanoparticles was studied against two bacterial strains, *S. aureus* and *E. coli*. The inhibition zones around the silver nanoparticles, silver nitrate and leaf extract disks on both bacterial strains was noted and measured after 24 h of incubation at 37°C. The synthesized silver nanoparticles proved to possess enhanced antibacterial activity against both *S. aureus* and *E. coli,* as clearly demonstrated by the clear zone of inhibition produced in Figures 11 and 12.

Also, the *C. anguria* leaf extract exhibited poor antibacterial activity when applied alone but there was an improved zone of clearance in combination with AgNPs. This shows the need for stabilization of AgNPs with leaf extract compounds. The lower antibacterial activity of the leaf extract could be due to its medium of extraction as well as due to lower concentration during experimentation.

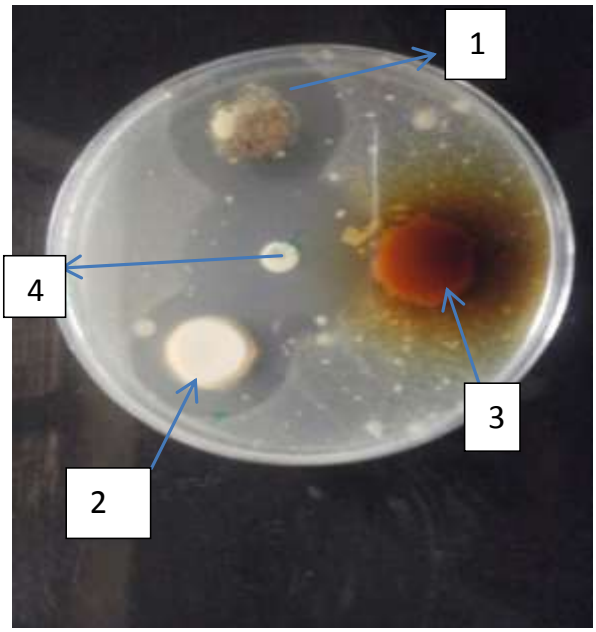

Figure 12. The antibacterial activity of (1) AgNPs, (2) $AgNO_3$, (3) leaf extract and (4) Kanamycin against *E. coli*.

Table 1. The antibacterial activity of AgNPs, $AgNO_3$, *C. anguria* leaf extract and reference drug against *S. aureus* and *E. coli*.

Name of bacterial species	Zone of inhibition (mm)			
	AgNPs	$AgNO_3$	Leaf extract	Kanamycin
S. aureus (Gram-positive)	11	7	2	15
E. coli (Gram-negative)	13	9	4	15

Thus, protocols for enhancing extracted concentration levels should be developed. The results of the antibacterial activity of the synthesized silver nanoparticles evaluated from the disc diffusion method are shown in Table 1.

The results obtained show that silver nanoparticles have a potential antimicrobial activity against both Gram-positive and Gram-negative bacteria which are comparable with both silver nitrate and the standard drug (kanamycin).

It can be noted from the results that the diameter of the inhibition zone is higher for the Gram-negative bacteria (*E. coli*) than the Gram-positive bacteria (*S. aureus*). This slight difference can exist as a result of the differences in the composition of their cell wall. Gram-negative bacteria cell membrane comprises of a single layer of peptidoglycan whereas Gram-positive bacteria cell membrane comprises of multi-layers of peptidoglycan that makes it more rigid for penetration (Roy et al., 2015). The bacterial cell wall is believed to possess small negative charge and as a result attract the silver cations from nanoparticles when they come closer to the bacterial cell wall. When Ag^+ ions experience some electrostatic attraction towards the bacterial cell wall, they move towards the cell wall and get attached to it and this leads to a change in the composition of the cell wall there by affecting its permeability (Ahmed et al., 2016). It is known that upon treatment with Ag^+ ions the microorganism DNA loses its replication ability and the expression of ribosome subunit proteins as well as other cellular proteins and enzymes essential to ATP production becomes inactivated. The antibacterial effect of the synthesized silver nanoparticles can be conferred by their extremely small size and their increased surface area through which they destroy the cell membrane, enter the microbe and cause intracellular damage (Franci et al., 2015).

Conclusion

The results of the present study suggest that *C. anguria* leaf extract can be used to synthesize silver nanoparticles with enhanced antibacterial activity which

makes them a potential source of antibacterial agent against *S. aureus* and *E. coli*. The synthesized silver nanoparticles were found to be spherical in shape, crystalline and face centered with an average diameter of between 11.01 and 27.0 nm. Formation time, pH and concentration of leaf extract were found to affect the formation of silver nanoparticles. An increase in pH was found to enhance nanoparticle formation and the optimum pH for formation of silver nanoparticles was pH 11. Maximum formation time for synthesis of silver nanoparticles was 48 h. The synthesis of silver nanoparticles enhances the therapeutic efficacy and medicinal values of *C. anguria*. Hence the results are promising and they prove to be an important step in the direction of medicine as it decreases the burden of multidrug resistance. Characterization techniques such as UV-vis, FTIR, TEM and XRD strongly suggest the synthesis of silver nanoparticles and the disc diffusion method proved the antibacterial activity of the synthesized silver nanoparticles.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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