OPEN ACCESS

African Journal of **Biotechnology**

8 August 2018
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJB

The African Journal of Biotechnology (AJB) is a peer reviewed journal which commenced publication in 2002. AJB publishes articles from all areas of biotechnology including medical and pharmaceutical biotechnology, molecular diagnostics, applied biochemistry, industrial microbiology, molecular biology, bioinformatics, genomics and proteomics, transcriptomics and genome editing, food and agricultural technologies, and metabolic engineering. Manuscripts on economic and ethical issues relating to biotechnology research are also considered.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journals of Biotechnology is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

All articles published by Academic Journals are licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.org

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editor-in-Chief

Editorial Board Members

Table of Content

Vol. 17(32), pp. 981-988, 8 August, 2018
DOI: 10.5897/AJB2017.16325
Article Number: 6F1D7FD58068
ISSN: 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Nuclear isolation and purification using SDS/urea (NIPSU) method for efficient and rapid extraction of high-purity genomic DNAs from *Jatropha curcas* L: A comparative analysis of DNA isolation protocols

Yoshihiko Nanasato[1,3,*], Ryo Uenoyama[1], Kaede Tomooka[1], Atsushi Kato[2,4] and Kinya Akashi[1,2*]

[1]School of Agricultural, Biological and Environmental Sciences, Faculty of Agriculture, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan.
[2]The United Graduate School of Agricultural Sciences, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan.
[3]Forestry and Forest Products Research Institute Forest Bio-Research Center, 3809-1 Ishi, Juo, Hitachi, Ibaraki 319-1301, Japan.
[4]Center for Sustainable Development, College of Arts and Sciences, Qatar University, Al Jamiaa St, Doha, Qatar.

Received 17 November, 2017; Accepted 20 July, 2018

***Jatropha curcas* L. (Jatropha) has gained popularity for its potential use in biodiesel production in arid regions, and its yield improvement by molecular breeding has been anticipated. However, Jatropha is known for its recalcitrance in the extraction of pure genomic DNAs. In this study, four DNA extraction methods were comparatively examined for their efficiency and quality of genomic DNA isolations from Jatropha. Consequently, one method, designated as nuclear isolation and purification using SDS/urea (NIPSU) method was found to be the most effective for rapid preparation of high yield and quality genomic DNA from Jatropha leaves. NIPSU method allowed extraction of genomic DNAs from leaves, stems, and roots tissues of Jatropha in high yield (6.8 to 24.5 µg gFW^{-1}), with spectroscopically high purity (A_{260}/A_{280} and A_{260}/A_{230} >1.8). NIPSU method was also applicable to the dehydrated leaves for genomic DNA preparations. The isolated genomic DNA was amenable for downstream molecular procedures such as polymerase chain reaction, restriction enzyme cutting and Southern blotting analyses.**

Key words: *Jatropha curcas* L., arid region, genomic DNA isolation, nuclear isolation, polymerase chain reaction, Southern blotting analysis.

INTRODUCTION

Jatropha *(Jatropha curcas* L.), a perennial shrub belonging to the Euphorbiaceae family, originated in Central America (Heller, 1996), but is now distributed widely over tropical and subtropical areas including Africa (Silitonga et al., 2013). Jatropha has been traditionally used as a hedge and a medicinal material. Recently, increasing concern regarding

*Corresponding author. E-mail: akashi.kinya@muses.tottori-u.ac.jp or nanasato@affrc.go.jp Tel/Fax: +81-857-31-5352.

#Authors contributed equally.

the global climate change and the depletion of fossil fuel reserves have been raised and Jatropha has received a considerable attention as a feedstock for renewable biodiesel fuel production. This species shows a number of advantageous traits, including its high yield of seed oil and resistance to adverse growing conditions such as drought, high salinity, and high temperature (Openshaw, 2000; Pandey et al., 2012). Elite varieties of Jatropha will be required for large-scale commercial production. However, Jatropha has a short history of breeding and is still essentially a wild plant; therefore, improved cultivars with stable yields in fluctuating environments remain to be developed. With the development of molecular breeding technology, various kinds of PCR-based molecular markers have been established for Jatropha, such as random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), single nucleotide polymorphism (SNP), inter simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP) (Basha and Sujatha, 2007; Sudheer Pamidimarri et al., 2009b; Wang et al., 2011; Kanchanaketu et al., 2012; Sillma et al., 2016). These technologies have the potential to assist in rapid breeding advances in Jatropha. However, isolation of high-purity genomic DNA from Jatropha is a challenging task because of the presence of a range of contaminants in the extracts, such as polyphenolics, polysaccharides and latex, which tend to be co-purified with DNAs during the conventional isolation procedures, and interfere with the reactions of DNA-modifying enzymes such as restriction enzymes and DNA polymerases (Kumar et al., 2003). Genomic DNA extraction from Jatropha tissues using commercially available kits often leads to low yield. Moreover, the cost for purchasing commercial kits should not be ignored. Cetyltrimethylammonium bromide (CTAB) is a cationic detergent that has a useful property of forming complexes with proteins and polysaccharides but not with nucleic acids at high ionic strength, and the CTAB-based methods have often been used to obtain genomic DNA from Jatropha tissues (Dhakshanamoorthy and Selvaraj, 2009; Sudheer Pamidimarri et al., 2009a; Mastan et al., 2012). However, the conventional CTAB-based methods require a time-consuming 30 to 90 min incubation step, which are accompanied by the labor-intensive manual procedures such as repeated phenol/chloroform extraction.

On the other hand, Liu et al. (1995) developed an isolation buffer containing SDS/urea and successfully applied to the extraction of genomic DNA from *Arabidopsis*. In this method, a strong anionic detergent SDS and a denaturant urea in the isolation buffer was allowed to extract genomic DNA without an incubation step, and the procedure was simplified by combining the procedures of DNA extraction and phenol/chloroform purification into single step. In other study, a one-step nuclear isolation procedure was successfully introduced to remove impurities from DNA-rich nuclear fraction in the plant extracts, using a nuclear isolation solution containing polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), and β-mercaptoethanol (Wagner et al., 1987). However, to the best of the authors' knowledge, effects of the nuclear isolation procedure and the SDS/urea isolation buffer on Jatropha DNA isolation has not been examined so far. Therefore, the objective of this study was to comparatively examine the efficiency of these procedures on DNA extraction from Jatropha tissues, and to optimize a rapid and efficient method for the DNA extraction for practical use in molecular analyses. The study also examined whether the optimized DNA extraction procedure could be applicable to ethanol-treated dehydrated tissue samples (Sharma et al., 2003), which has been developed for stabilizing tissues for longer storage periods without cooling condition in large-scale field experiments.

MATERIALS AND METHODS

Reagents

Ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt (EDTA) was purchased from Dojindo Laboratories (Kumamoto, Japan). Chloroform, ethanol, and sodium chloride were from Wako Pure Chemical Industries (Osaka, Japan). Polyethylene glycol #6000 (average molecular weight: 7,400 to 10,200) and other reagents were from Nacalai Tesque (Kyoto, Japan).

Plant materials

Jatropha curcas L. variety IP-3P was obtained from Pakuwon Station, Indonesian Center for Estate Crop Research and Development, Sukabumi, West Java, Republic of Indonesia. For evaluation of various DNA extraction methods, fully-expanded fresh leaves collected from Jatropha trees of 1 to 2 m height grown in a greenhouse were used. For comparison of DNA extraction efficiency in leaf, stem, and root tissues, juvenile Jatropha seedlings at a height of 20 to 25 cm were used, which were grown in a growth room at 25 to 28°C with a 14 h/10 h (light/dark) photoperiod using white fluorescent lamps (150 to 170 µmol photons m^{-2} s^{-1}, Plantlux, Toshiba Lightning and Technology, Tokyo, Japan).

Dehydration of leaf tissues was performed by detaching a leaf from the Jatropha plants grown in the greenhouse, submerged immediately in 40 mL of absolute ethanol in a 50 mL tube, and incubated at room temperature for 30 to 60 min. The ethanol-treated leaf samples were then allowed to air dry. Dehydrated leaves were kept at room temperature for short-term storage, or at -30°C for longer preservation.

Evaluation of DNA extraction methods

To compare the methods for DNA extraction from Jatropha, the following four methods were examined. In each method, a 50 to 80 mg sample of fresh tissue was ground to a fine powder using a mortar and pestle under liquid nitrogen, then used for the genomic DNA extractions. In each method, the extractions were repeated four times using four different Jatropha samples.

Method I

This method was a CTAB-based protocol essentially in accordance

with the report by Murray and Thompson (1980), with an attached modification related to the DNA precipitation procedure. After the CTAB-DNA precipitation, the pellet was dissolved in 400 µL of 1.0 M CsCl. The 800 µL of absolute ethanol was added and mixed by gentle inverting, then incubated at -20°C for 20 min. The precipitate was recovered by centrifugation at 15,000 × *g* for 10 min at 4°C. After discarding the supernatant, the pellet was washed with 70% ethanol, then dissolved in TE buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Method II

The method was a SDS/urea-based protocol essentially in accordance with the report by Liu et al. (1995), with a minor modification to the extraction buffer, which was composed of 50 mM Tris-HCl (pH 7.5), 20 mM EDTA, 0.3 M NaCl, 0.5% SDS, 5 M urea, 5% (v/v) TE-saturated phenol, and 10 mM β-mercaptoethanol.

Method III

This method was a combination of a procedure of nuclear isolation followed by the method I described previously. For the nuclear isolation procedure, 1 mL of ice-cold nuclear isolation buffer containing 10% PEG #6000, 5% PVP K-30 (average molecular weight 40,000), 0.35 M sorbitol, 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5% β-mercaptoethanol was added to the ground tissues and vortexed vigorously. The suspension was centrifuged at 15,000 × *g* for 10 min at 4°C. After discarding the supernatant, genomic DNA was extracted from the pellet using the procedure in the method I described previously.

Method IV (NIPSU: nuclear isolation and purification using SDS/urea)

This method was a combination of the nuclear isolation procedure described in the method III, followed by the modified method II described previously. After the nuclear isolation treatment described in the method III, the pellet was dissolved in 500 µL of extraction buffer (described in the method II) and vortexed vigorously. After centrifuging at 15,000 × *g* for 10 min at room temperature (20 to 25°C), the supernatant was transferred to a new tube and centrifuged again using same condition to remove insoluble impurities. The supernatant was transferred to a new tube, and one volume (that is, 500 µL) of TE-saturated phenol: chloroform: isoamyl alcohol=25:24:1 (PCI) was added and mixed gently by inverting the tube. After centrifuging at 15,000 × *g* for 5 min at room temperature, the aqueous phase was transferred to a new tube. Two volumes of ethanol (that is, 1 mL) was added to the supernatant and centrifuged at 15,000 × *g* for 5 min at 4°C. The pellet was washed with 1 mL of 70% ethanol and repeated the same centrifugation step. DNA was dissolved in TE buffer.

Analysis of the spectroscopic property and quantity of genomic DNA

The ratios of optical absorbance at 260 and 280 nm (A_{260}/A_{280}), and of 260 and 230 nm (A_{260}/A_{230}) were used to assess the purity of genomic DNAs using a spectrophotometer (WPA Biowave S2100, Biochrom, Cambridge, UK). Quantity of genomic DNA was estimated by gel electrophoresis analysis with GelAnalyzer 2010 software (http://www.gelanalyzer.com), using a dilution series of *Sty*I-digested lambda DNA with known concentration as the calibration standards.

Polymerase chain reaction

Primers specific for the actin gene (Nanasato et al., 2015, 5'-AGACCTCCAAAACCAGCTCA-3' and 5'-TTGATTTTCATGCTGCTTGG-3'), 620 bp of the curcin promoter region (GenBank accession number AF469003, 5'-CTCGAGAATATTGGAATAGAAGACTTTG-3' and 5'-TCTAGACAAATATCATTATACGAATACG-3', *Xho*I and *Xba*I sites are indicated as underlines, respectively), and 5,956 bp of *JcDGAT1* (contig number Jcr4S00005, Jatropha genome database; Sato et al., 2011), 5'-ATGACGATTTTGGAGACCACTACTA-3' and 5'-TCATCTTAATTCAGCATTGCCTTTC-3') were used for amplification in 10 µL reaction volumes containing Jatropha genomic DNA as the template. PCR amplification was performed using ExTaq DNA polymerase (Takara Bio, Shiga, Japan) using the following conditions: 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C (*JcDGAT1*), 58°C (actin) or 63°C (curcin promoter) for 30 s, and 72°C for 1 min (actin and curcin promoter) or 6 min (*JcDGAT1*); and a final extension of 72°C for 7 min. PCR products were separated on a 0.8% agarose gel, and visualized by GelGreen Nucleic Acid Gel Stain (Biotium, Fremont, CA).

Southern blotting analysis

Genomic DNA (5 µg) was digested with *Eco*RV or *Hin*dIII, separated on a 0.8% agarose gel, then transferred to a positively charged nylon membranes (Pall, Port Washington, NY) with 20× saline sodium citrate buffer as described previously (Sambrook and Russell, 2001). A digoxigenin (DIG)-labeled DNA probe specific for the *JcDGAT1* coding sequence was prepared by amplifying 328 bp of *JcDGAT* cDNA fragment from a cDNA clone of *JcDGAT* by PCR using a pair of primers (5'-ATGACGATTTTGGAGACCACTACTA-3' and 5'-CCCTGAGCGCTCGATGAG-3'), and used as a template for the probe synthesis using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Penzberg, Germany), and hybridized bands were then detected using X-ray film (Hyperfilm ECL, GE Healthcare, Buckinghamshire, UK).

Statistical analysis

One-way analysis of variance (ANOVA) was adopted for the data analysis. Means were separated by Tukey's honest significant difference (HSD) to identify significant differences ($P < 0.05$) between the samples.

RESULTS AND DISCUSSION

Evaluation of different DNA extraction methods for Jatropha leaves

Efficiency of four DNA extraction methods, that is, methods I to IV, was evaluated using fresh leaves of Jatropha. Agarose gel electrophoresis showed multiple band signals form the extracted DNAs, which were composed of a single band corresponded to the genomic DNAs which migrated at a similar speed with 19.3-kbp DNA size marker, and three bands migrated with 0.42 to 1.88 kbp markers which showed a characteristic pattern for ribosomal and transfer RNAs (Figure 1a). Quantification of the genomic DNAs by image analysis showed that the method I, which was a conventional CTAB method, gave a lowest average yield of 1.12 ± 0.16

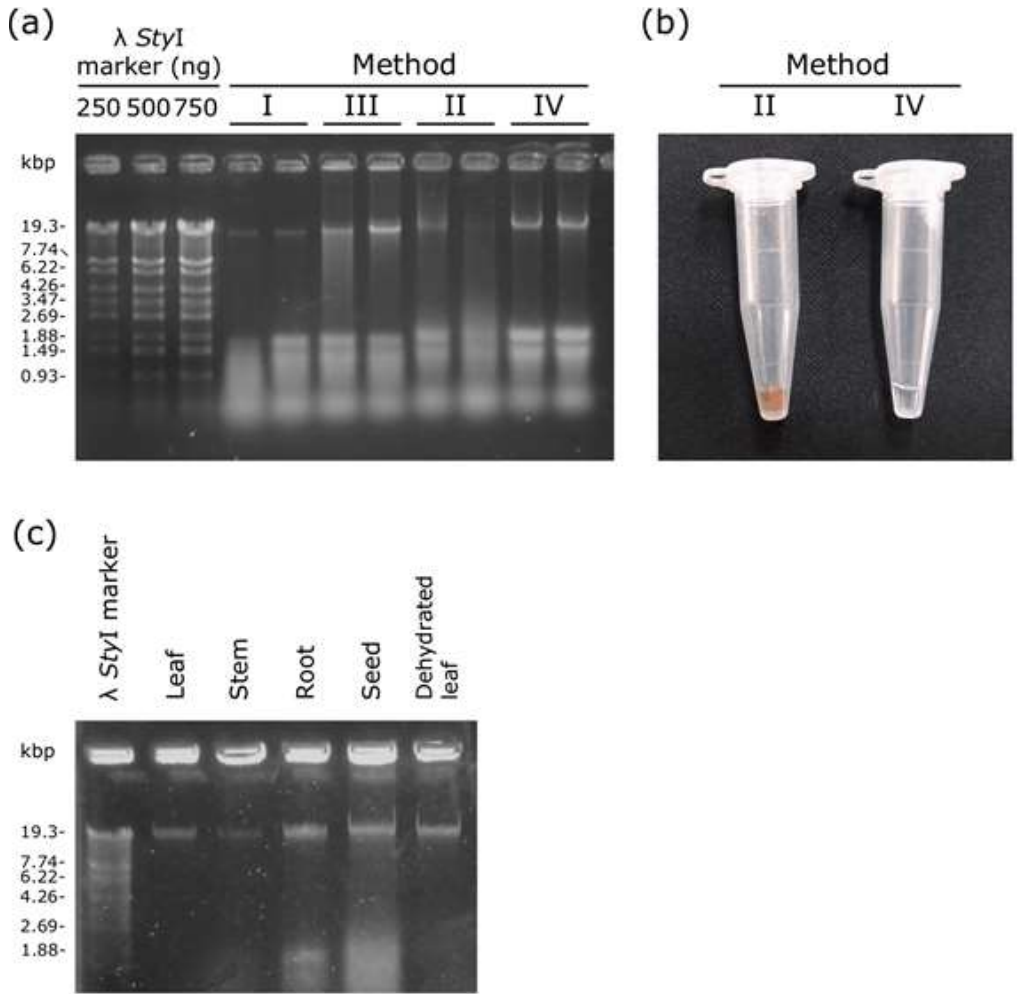


Figure 1. Properties of Jatropha genomic DNAs isolated by the four methods in this study. (a) Agarose gel electrophoresis analysis of genomic DNAs extracted by the methods I to IV. The amount of DNA loaded into each well corresponded to that extracted from 13.0 to 18.2 mg of fresh leaf tissues. Two independent DNA sample preparations were loaded for each method. Lane M indicates *Sty*I-digested lambda phage DNA marker, and values at the top of each lane indicate quantity of the 19.3 kb fragment in nanograms. (b) Coloring of the genomic DNA solutions extracted by the method II (left). Transparent DNA solution extracted by the method IV (NIPSU) is also shown for comparison (right). (c) Agarose gel electrophoresis analysis of genomic DNAs isolated from various tissues of Jatropha using the NIPSU method. The amount of DNA loaded into each well corresponded to that extracted from 20.8 to 63.0 mg of fresh tissues

μg g FW^{-1} among the four methods tested in this study (Table 1). DNA yield of method II, a SDS/urea-based method, was 7.22 ± 4.67 μg g FW^{-1}, but the yields were highly variable among four independent extractions as suggested by the large standard deviation. A brownish color was observed in the DNA solution obtained by the method II (Figure 1b), showing the presence of impurities in this preparation. This observation suggested that the method II was not effective for eliminating inhibitory compounds for downstream molecular analyses. Introducing the nuclear isolation procedure in methods III and IV contributed greatly to the removal of impurities, and drastically improved the extraction efficiency. The amount of genomic DNAs obtained by the methods III and IV were 9.32 ± 2.69 and 7.93 ± 0.86 μg g FW^{-1}, respectively, and were significantly higher than that in the method I. Cumulative total time required for the incubation and centrifugation steps was 140 and 50 min for the method III and IV, respectively, showing that the method III was more time-consuming than the method IV. Therefore, it is judged that the method IV, hereinafter designated as nuclear isolation and purification using SDS/urea (NIPSU) method, was the most convenient protocol for extracting genomic DNA from fresh Jatropha leaves. The whole scheme of the NIPSU method is shown in Figure 2. From this point onward, only the NIPSU

Table 1. Yield of genomic DNA extraction using the Methods I to IV.

Method	Major buffer components	Nuclear isolation procedure	Yield (µg g FW^{-1})[1,2]
I	CTAB	-	1.12 ± 0.16^{a}
II	SDS/urea	-	7.22 ± 4.67^{ab}
III	CTAB	+	9.32 ± 2.69^{b}
IV (NIPSU)	SDS/urea	+	7.93 ± 0.86^{b}

[1] The unit is µg DNA per g fresh weight of leaf samples; [2] Each value represents the mean ± SD from four independent experiments. Statistical analyses were conducted by a one-way analysis of variance (ANOVA), and means were separated by Tukey's Honest Significant Difference (HSD). Values with significant difference were labeled with different letters ($P < 0.05$). CTAB, Cetyltrimethylammonium bromide; SDS, Sodium dodecyl sulfate.

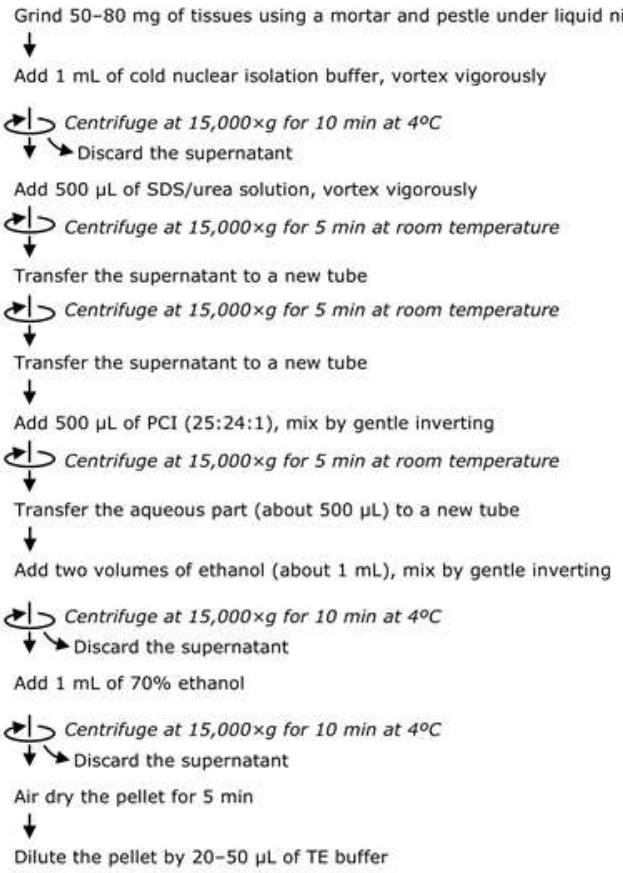


Figure 2. A schematic procedure of the NIPSU method for the extraction of genome DNA from Jatropha tissues used in this study. PCI, Phenol-chloroform-isoamyl alcohol (25:24:1).

method was used for further analyses in this study.

Evaluation of the NIPSU method in various tissues and dehydrated leaf

Subsequently, whether the NIPSU method was effective to non-leaf tissues in Jatropha was examined. Consequently, agarose gel electrophoresis analysis showed that genomic DNA preparations extracted from stem, roots, and seeds gave a band signal corresponding to the high molecular weight DNAs (Figure 1c), suggesting that the NIPSU method was also applicable to non-leaf tissue. Yields of genomic DNA were in the range 6.82 to 26.46 µg g FW^{-1} (Table 2). The yields from stem and root tissues were lower than that from leaves, which was consistent with previous report (Sudheer Pamidimarri et al., 2009a). Spectrophotometer analysis

Table 2. Yield and spectroscopic properties of genomic DNAs from various tissues extracted by the Method IV (NIPSU method).

Tissue type	Yield (µg gFW^{-1})[1,2]	A_{260}/A_{280}[2,3]	A_{260}/A_{230}[2,3]
Leaf	24.51 ± 5.86[a]	1.97 ± 0.079[c]	2.04 ± 0.16[de]
Stem	6.82 ± 1.67[b]	2.03 ± 0.044[c]	2.15 ± 0.33[d]
Root	6.97 ± 2.52[b]	1.95 ± 0.051[c]	1.89 ± 0.092[de]
Seed	26.46 ± 7.69[a]	1.75 ± 0.27[c]	1.19 ± 0.49[e]
Dehydrated leaf	9.22 ± 2.90[b]	2.11 ±0 .62[c]	1.26 ± 0.37[e]

[1]The unit is µg DNA per gram fresh weight of tissue samples. [2]Each value represents the mean ± SD from four independent experiments. Analyses were conducted as a one-way ANOVA and means were separated by Tukey's HSD. Values with significant difference were labeled with different letters ($P < 0.05$). [3]A_{230}, A_{260}, and A_{280} are the optical absorbance at wavelengths of 230, 260, and 280 nm, respectively, and their ratio were presented as the indicators of quality of DNA preparations.

revealed that the spectroscopic indicators of DNA purity, A_{260}/A_{280} and A_{260}/A_{230} ratios, of genomic DNAs extracted from leaf, stem, and roots were in the range of 1.89 to 2.15, suggesting that these DNA preparations were highly pure (Table 2). Relatively low A_{260}/A_{230} value of 1.19 ± 0.49 was recorded for genomic DNA from seeds, which might be due to the high abundance of organic contaminants such as carbohydrates in this tissue.

The NIPSU method was then applied to dehydrated Jatropha leaves. Dehydration treatment of the leaves using ethanol resulted in the significant decrease in the sample weight, and the ratio of dry weight/fresh weight was 0.19 ± 0.066 (n = 4). This value was similar to that in previous reports (Patel et al., 2010; Nahar and Hoque, 2013). From the dehydrated leaves, 9.22 ± 2.90 µg g FW^{-1} of genomic DNA was obtained with spectroscopic properties (2.11 ± 0.62 and 1.26 ± 0.37 for A_{260}/A_{280} and A_{260}/A_{230} ratios, respectively; Table 2), and the DNA intactness was confirmed by agarose gel electrophoresis (Figure 1c), suggesting that the NIPSU method was applicable to dehydrated leaf tissues.

Molecular analyses using genomic DNAs extracted by the NIPUS method

To examine the behavior of DNAs extracted by the NIPUS method in the molecular analyses, PCR and restriction assays were performed. In the PCR experiments, three representative DNA fragments, that is, actin coding sequence (301 bp), curcin promoter (632 bp), and *JcDGAT1* coding sequence (5,956 bp) were amplified from the DNA isolated from either fresh or dehydrated leaves. Gel electrophoresis analysis showed clear, sharp single bands with expected sizes, demonstrating that all PCR reactions were successful in amplifying the target gene fragments (Figure 3a). The suitability of the isolated genomic DNA for downstream enzymatic procedures was also determined by Southern blotting analysis using a probe for a single gene *JcDGAT1* (Figure 3b). Consequently, a single band corresponding to *JcDGAT1* gene was clearly detected from only 5 µg of *Eco*RV- and *Hin*dIII-digested genomic DNA. These results show that genomic DNAs extracted by the NIPSU method was compatible to PCR amplification and restriction analyses, and can be useful for various molecular analyses in the future, for example, RFLP, RAPD and SSR analyses.

Conclusion

Four methods were comparatively evaluated for the efficiency of DNA extraction from fresh leaves of Jatropha, and one of these methods, NIPSU (nuclear isolation and purification using SDS/urea), was found to be the most effective and fast method for extraction of genomic DNA from Jatropha. This method was applicable to a wide range of Jatropha tissues and dehydrated leaves, and amenable for downstream molecular analyses such as PCR and restriction analysis.

CONFLICT OF INTERESTS

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Science and Technology Research Partnership for Sustainable Development (SATREPS) program from the Japan International Cooperation Agency (JICA) and the Japan Science and Technology Agency (JST), and the New Energy and Industrial Technology Development Organization (NEDO). The authors acknowledge funding from the Grant-in-Aid for Scientific Research (17K07755) from JSPS, and by the Joint Research Program and the Project Marginal Region Agriculture, the Arid Land Research Center, Tottori University, and the International Platform for Dryland Research and Education (IPDRE) Program, Tottori University.

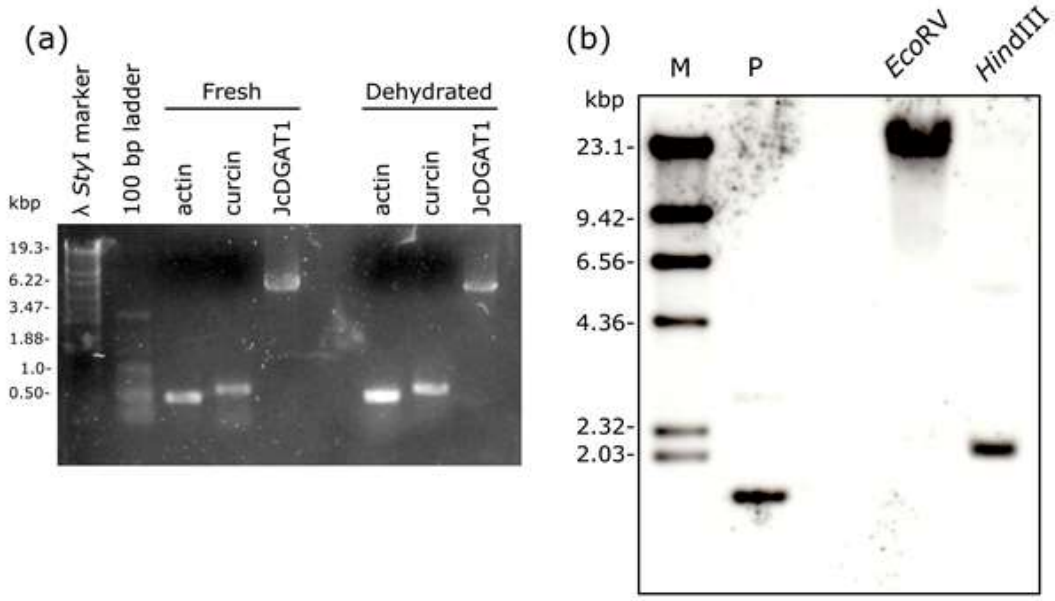


Figure 3. Molecular analyses of the Jatropha genomic DNAs extracted by the NIPSU method. (a) PCR amplification of representative DNA fragments from Jatropha genomic DNAs. Genomic DNAs isolated from fresh or dehydrated leaves was used as templates to amplify the fragments for coding sequence of *JcActin* (actin), promotor region of *JcCurcin* (curcin), and coding sequence of *JcDGAT1,* and visualized by an agarose gel electrophoresis. (b) Southern blotting analysis of *JcDGAT1* gene. The 5 µg of genomic DNA isolated from fresh leaves was digested using *Eco*RV or *Hin*dIII restriction enzymes, separated on a 0.8% agarose gel, then transferred to a nylon membrane. The membrane was hybridized with a DIG-labeled *JcDGAT1* probe and visualized. Lane M, DIG-labeled λ/*Hin*dIII DNA marker. Lane P, *Xba*I-digested *JcDGAT* cDNA in a pBluescriptII-SK+ plasmid as the positive control.

REFERENCES

Basha SD, Sujatha M (2007). Inter and intra-population variability of *Jatropha curcas* (L.) characterized by RAPD and ISSR markers and development of population-specific SCAR markers. Euphytica 156:375–386. doi: 10.1007/s10681-007-9387-5

Dhakshanamoorthy D, Selvaraj R (2009). Extraction of genomic DNA from *Jatropha* sp. using modified CTAB method. Romanian Journal of Biology Plant Biology 54:117-125

Heller J (1996). Physic nut. *Jatropha curcas* L. Promoting the conservation and use of underutilized and neglected crops. 1. Institute of Plant Genetics and Crop Plant Research, Gatersleben/ International Plant Genetic Resources Institute, Rome. ISBN: 92-9043-278-0

Kanchanaketu T, Sangduen N, Toojinda T, Hongtrakul V (2012). Genetic diversity analysis of *Jatropha curcas* L. (Euphorbiaceae) based on methylation-sensitive amplification polymorphism. Genet. Mol. Res. 11:944-955. doi: http://dx.doi.org/10.4238/2012.April.13.2

Kumar A, Pushpangadan P, Mehrotra S (2003). Extraction of high-molecular-weight DNA from dry root tissue of *Berberis lycium* suitable for RAPD. Plant Molecular Biology Reporter. 21:309a-309d. doi: 10.1007/BF02772807

Liu Y-G, Mitsukawa N, Oosumi T, Whittier RF (1995). Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant Journal 8:457-463. doi: 10.1046/j.1365-313X.1995.08030457.x

Mastan SG, Sudheer PDVN, Rahman H (2012). Molecular characterization of intra-population variability of *Jatropha curcas* L. using DNA based molecular markers. Molecular Biology Reports 39:4383-4390. doi: 10.1007/s11033-011-1226-z

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8:4321-4326. doi: 10.1093/nar/8.19.4321

Nahar K, Hoque S (2013). A Morphological and Physiological Study of *Jatropha curcas* Linn . Propagated from Seeds in Bangladesh. Middle-East Journal of Scientific Research 13:1115-1118. doi: 10.5829/idosi.mejsr.2013.13.8.623

Nanasato Y, Kido M, Kato A (2015). Efficient genetic transformation of *Jatropha curcas* L. by means of vacuum infiltration combined with filter-paper wicks. In Vitro Cellular & Developmental Biology-Plant 51:399-406. doi: 10.1007/s11627-015-9703-z

Openshaw K (2000). A review of *Jatropha curcas*: An oil plant of unfulfilled promise. Biomass Bioenergy 19:1-15. doi: 10.1016/S0961-9534(00)00019-2

Pandey VC, Singh K, Singh JS, et al (2012). *Jatropha curcas*: A potential biofuel plant for sustainable environmental development. Renewable. Sustainable. Energy Reviews 16:2870-2883. doi: 10.1016/j.rser.2012.02.004

Patel AD, Panchal NS, Pandey IB, Pandey AN (2010). Growth , water status and nutrient accumulation of seedlings of *Jatropha curcas* L. (Euphorbiaceae) in response to soil salinity. An. Biology 32:59-71

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harb Lab Press Cold Spring Harb NY P999. ISBN: 0879695773

Sato S, Hirakawa H, Isobe S (2011). Sequence analysis of the genome of an oil-bearing tree, *Jatropha curcas* L. DNA Research 18:65-76. doi: 10.1093/dnares/dsq030

Sharma R, Mahla HR, Mohapatra T, (2003). Isolating plant genomic DNA without liquid nitrogen. Plant Molecular Biology Reporter 21:43–50. doi: 10.1007/BF02773395

Silitonga AS, Masjuki HH, Mahlia TMI (2013). A global comparative review of biodiesel production from *Jatropha curcas* using different homogeneous acid and alkaline catalysts: Study of physical and chemical properties. Renew. Sustain. Energy Reviews 24:514-533. doi: 10.1016/j.rser.2013.03.044

Sillma R, Daneshwar P, Subhasisa B, Rajesh J (2016). Application of

rep-PCR as a molecular tool for the genetic diversity assessment of *Jatropha curcas*. African Journal of Biotechnology 15:172-179. doi: 10.5897/AJB2015.15008

Sudheer Pamidimarri DV, Boricha G, Reddy MP (2009a). A simplified method for extraction of high quality genomic DNA from *Jatropha curcas* for genetic diversity and molecular marker studies. Indian Journal of Biotechnology 8(2):187-192

Sudheer Pamidimarri DV, Singh S, Mastan SG, Patel J, Reddy MP (2009b). Molecular characterization and identification of markers for toxic and non-toxic varieties of *Jatropha curcas* L. using RAPD, AFLP and SSR markers. Molecular Biology Reports 36:1357-1364. doi: 10.1007/s11033-008-9320-6

Wagner DB, Furnier GR, Saghai-Maroof MA (1987). Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proceedings of the National Academy of Sciences USA 84:2097–2100. doi: 10.1073/pnas.84.7.2097

Wang CM, Liu P, Yi C(2011). A first generation microsatellite- and SNP-based linkage map of *Jatropha*. PLoS One 6:4-11. doi: 10.1371/journal.pone.0023632

Vol. 17(32), pp. 989-993, 8 August, 2018
DOI: 10.5897/AJB2018.16535
Article Number: 4C638CF58070
ISSN: 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluation and comparison of staining effect of *Punica granatum* flower extract on testis and ovary of Wistar rats: First results

Nilgün Güler Kuşçulu

Department of Chemistry Technology, Mustafa Cıkrkcoglu Vocational School, Erciyes University, TR-38039, Kayseri, Turkey.

Received 29, May, 2018; Accepted 30 July, 2018

The evaluation and comparison of the staining effect of pomegranate (*Punica granatum*) flower extract (dye solution) on histological sections of rat testis and ovary provides a simple, quick technique for the visualisation of both cells. In the developed procedure, this work shows the staining effect of *P. granatum* flower extract used at different pH of dye bath and temperatures on the histological sections of rat testis and ovary. A 20% stock solution of *P. granatum* flower extract was prepared by dissolving 20 g of the dye in 100 mL ethanol at room temperature for several hours. After 24 h, the deep-red coloured solution was filtered (0.45 µm filter) to remove any undissolved dye. This stock solution was stored at room temperature and each day a 5 mL sample was taken and adjusted to different pH with dilute ammonia (NH_3) using pH meter. Best staining effects conditions were obtained by testing the different pH of dye (1-2, 4-5) and temperature. The testis and ovary are stained in different colors; lamina propria section and spermatogonia cells of testis were more purple than the same sections of ovary at room temperature and pH of 1-2 dye bath. Therefore, the developed method has been applied successfully for the visualization of rat testis and not the ovarian tissue.

Key words: Histological section, ovarian, pomegranate, *Punica granatum,* testis**.**

INTRODUCTION

Natural dyes are environmental friendly. They exist in a lot of plants (Hu et al., 2017). For example, pomegranate is an important fruit crop of tropical and subtropical regions (Chandra and Babu, 2010). The flowering habit of pomegranate is influenced by the climatic condition of the geographical region where it is grown (Babu, 2010). Plants and their extracts are used for medicinal purpose both for the prevention and treatment of human diseases (Zeliha et al., 2016). Pomegranate extract is widely used for the treatment of some diseases in health sciences. For example, olive anthracnose is caused by different species of *Colletotrichum* spp. and may be regarded as the most damaging disease of olive fruits worldwide. A pomegranate peel extract (PGE) is very effective in controlling this disease (Pangello et al., 2017; Davulcu et al., 2014). Pomegranate peel extract decreases the damage induced by testis torsion (Beigi et al., 2017).

Natural compounds are important resources of many anticancer drugs. Pomegranate is a kind of antioxidant-rich fruit. Its peel and seed have potential anticancer

E-mail: nguler@erciyes.edu.tr.

activities. The extract from *Punica granatum* (pomegranate) peel induces apoptosis and impairs metastasis in prostate cancer cells. Prostate cancer is a big threat to males due to its poor prognosis and high mortality rate (Deng et al., 2017). In contrast to pomegranate or its peel, pomegranate flower is less used in health studies. For example, *P. granatum* flower has played an important role in the treatment of diabetes in herbal medicine (Xu et al., 2017). PG could be used as a dietary supplement in the treatment of chronic diseases characterized by atherogenous lipoprotein profile, aggravated antioxidant status and impaired glucose metabolism and also in their prevention (Bagri et al., 2009).

Constituents of the flowers of *P. granatum* were determined by spectroscopic analysis (Wang et al., 2006). Six known compounds were identified by comparing their spectral data with values reported in the literature as ellagic acid, 3, 3',4'- tri-O-methylellagic acid, ethyl brevifolin carboxylate, urolic acid, maslinic acid, and daucosterol (Wang et al., 2010; Mahmoud et al., 1994). According to their sources, dyes are divided into two classes as natural and synthetic (Nagar et al., 2005). Dyes from liquids in which they are completely or at least partially soluble are applied to different materials like textiles, leather, paper, hair, food, cells at different temperatures and time (Zollinger, 2004; Guler and Benli, 2017). Mostly in histological studies the most important and used dye is hematoxylin. It is used to demonstrate general tissue structures (Avwioro, 2002). This is the first study where pomegranate flower extract is used on testis and ovarian tissues. The aim is to elucidate the differences that occur when we dye testis and ovarian tissues under the same conditions, to reveal the positive and negative results of dyes and to direct future studies.

MATERIALS AND METHODS

Flowers of *P. granatum* were obtained from local markets of Kayseri. In this study, two Wistar albino male and female rats weighing 150-250 g were obtained from Erciyes University Experimental and Clinical Research Center (DECAM) and used according to the decision of the ethics of the committee 16/144 dated 16.11.2016.

Preparation of the extract by cold waiting process in ethanol

Dried flowers of *P. granatum* were ground into a dark red-black powder using manual grinding machines (Waring, commercial). The dry powder of the plant weighed 20 g (Schimadzu bl 3200). The powder was kept in 100 mL ethanol for 24 h at room temperature. Then the extract (20 % w/v) was filtered two times by blue filter paper. The filtrate was stored in the refrigerator at 4°C and then was used for staining.

Preparation of sections

Testis and ovarian tissues were carefully dissected, trimmed of all fat, and blotted dry to remove any blood. They were fixed in 10% formal saline. The fixed tissues were transferred through a graded series of ethanols. On day 1, they were placed in 70% alcohol for 7 h, then transferred to 90% alcohol and left in the latter overnight. On day two, the tissues were passed through three changes of absolute alcohol for 1 h each, then cleared in xylene. The tissues were infiltrated with three changes of molten parafin wax at 1 h intervals in an oven at 58°C. The tissues were embedded in wax and blocked embedding. The specimens were oriented so that sections would be cut perpendicular to the long axis of the testis and ovary. These sections were designated vertical sections. Serial sections of 5 µm thick were obtained from a block using a rotary microtome, they were attached to slides and dried at 65°C for 45 min.

Staining method

A 300 mL *P. granatum* flower extract (20% w/v, stain solution) was distributed to the four 80 mL beakers in portions of 75 mL. Two of the beakers were left empty, and pH (1-2) of the solutions was measured until pH of 4-5 was obtained. The NH_3 solution was added to the other two beakers. They were mixed with glass stirrer. Prepared testis and ovary were submerged into the four beakers. At the first stage, the first and third beakers were left at room temperature for an hour. An hour later, the slides were removed from the stain solutions and washed with distilled water, and left to air-dry. At the second stage, the second and fourth beakers were placed in the oven at 100°C for one hour. An hour later, the slides were removed from the dye solutions and washed with distilled water, and left to air-dry. Photographs of the testis and ovarian tissues on the eight slides were taken at 10×50 magnification by light microscopy (Olympus BX-51, Japan).

RESULTS

Testis and ovarian tissues were stained in pomegranate flower extract. Acidity and dyeing temperature were changed in the extract. Dyeing results of the tissues are given in Figure 1. We examined and compared the dyeing results of the tissues. More different regions, cells and erythrocytes were stained with green, brown, light red colors in ovarian tissue at 100°C dyeing temperature. This is seen in Figure 1a, tissue surface was stained with light purple-green at room temperature in Figure 1b.

Testis tissue was stained in different colors at different temperatures and dye solution pH of 4-5. Epithel cells in testis tissue were stained in brown color at 100°C as seen in Figure 2a. But, the same cells have very light green color at room temperature as seen in Figure 2b. When the pH of dye solution was changed, tissue surfaces were stained in different colors as seen in Figure 3a, b and c. As seen in Figure 3b, best dyeing color of spermatogonia and sertoli cells appeared at pH 1-2 or in more acidic dye solution as purple. Because cell nucleus contains histon proteins which are base, strong acidic dye molecules interact with base protein molecules making staining to occur. Comparing the pictures of testis and ovarian tissues in Figures 5a and 4a, testis tissue is stained as purple color more than ovarian tissue at pH 1-2 in strong acidic dye solution. Ovarian and testis tissues have light green-yellow color at pH 4-5 in weak acidic dye solution. This is seen in Figures 4 c and 5c.

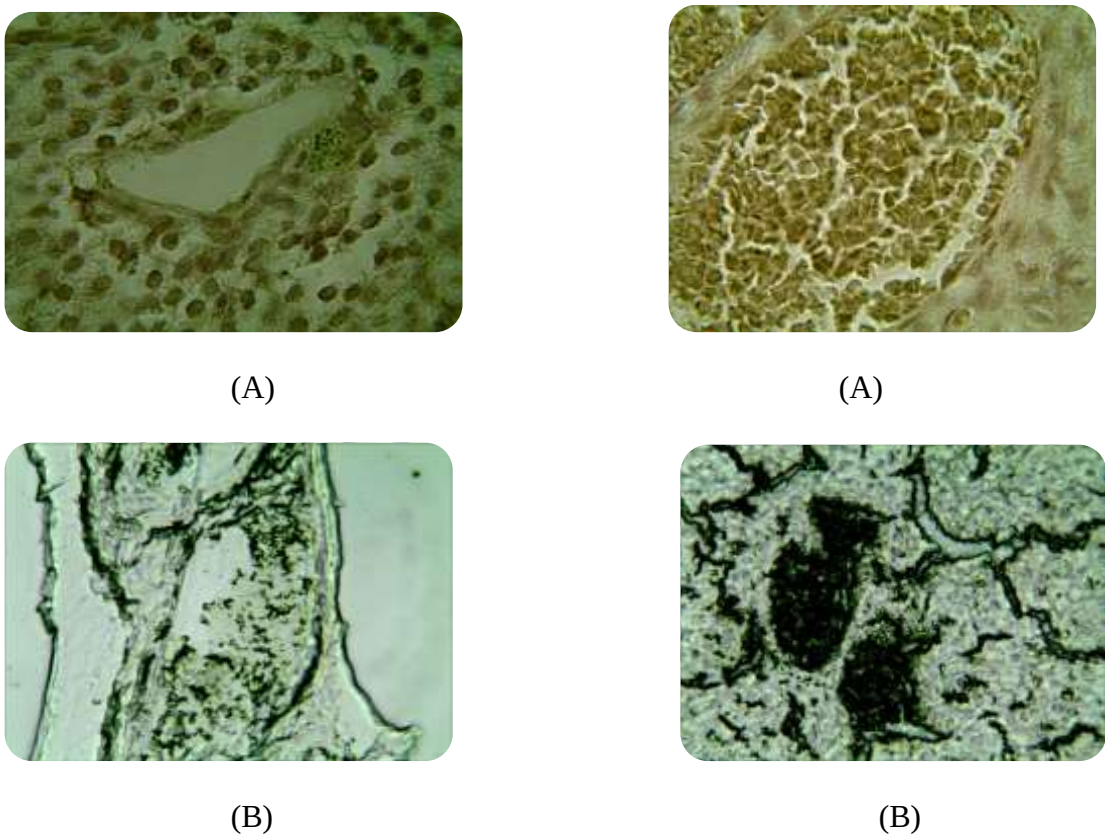


Figure 1. Photographs of the ovarian tissue at pH value 4-5 for one hour in pomegranate flower extract ×50 A) at 100°C, B) at room temperature.

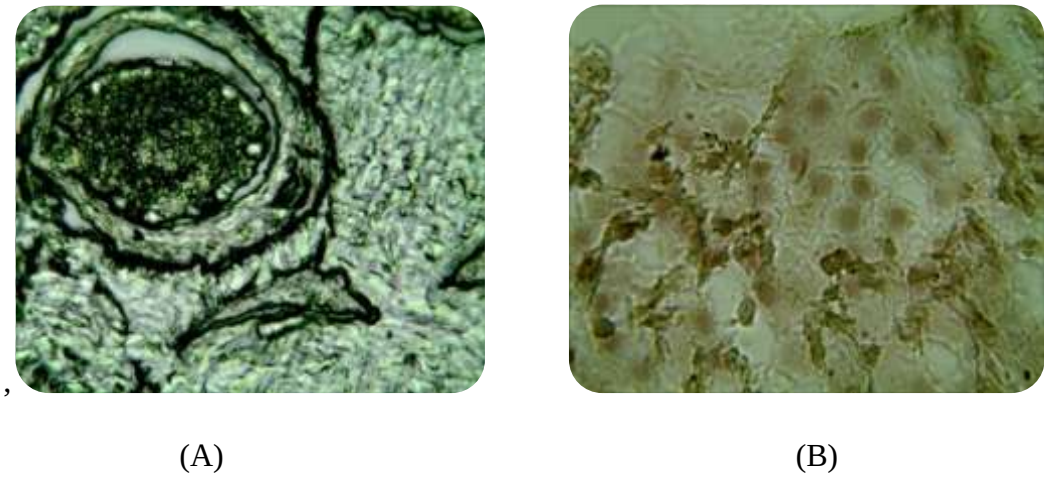
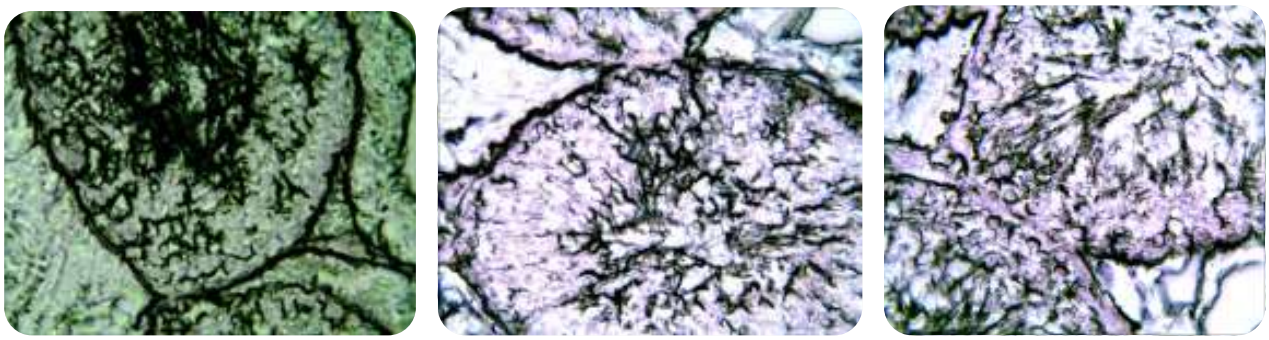


Figure 2. Photographs of the testis tissue at pH 4-5 for one hour in pomegranate flower extract ×50 A) 100°C, B) at room temperature.

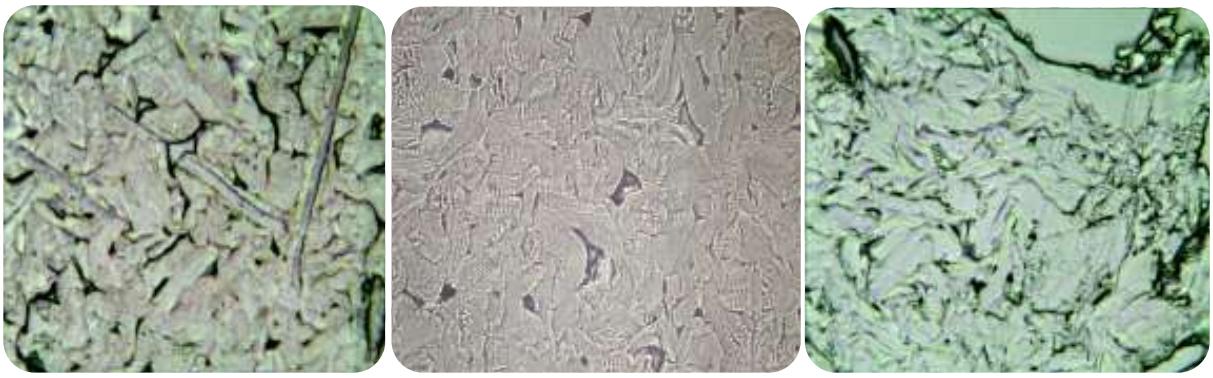
DISCUSSION

Our study focused on the use of non-allergenic, non-toxic and ecofriendly natural pigment for histology. Bassey et al. (2012) also used ethanol solvent from a family of hibiscus only to stain testis tissue. The pH value of the extract is in the range of 4-5. They applied the extract to the tissue in varying amounts and durations at constant



(A) (B) (C)

Figure 3. Photographs of testis tissue at different pH, at room temperature in pomegranate flower extract ×50 A) pH 4-5, B) pH 1-2.



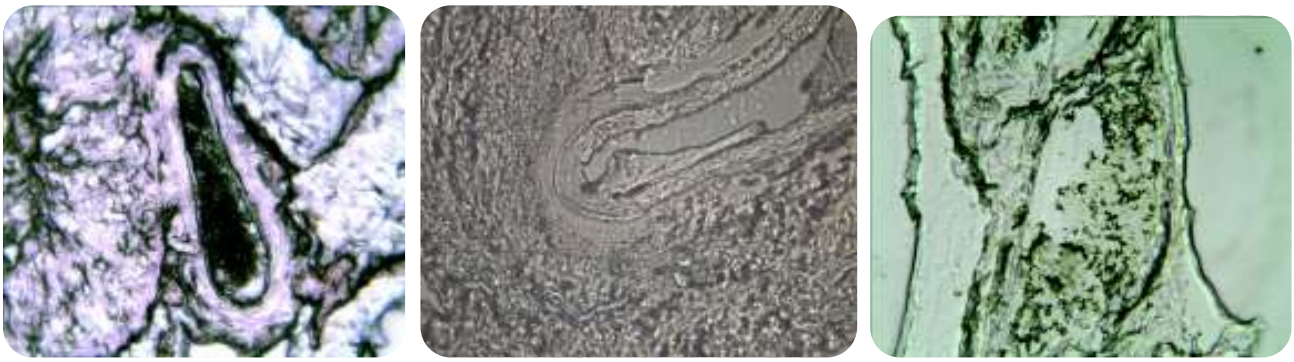
(A) (B) (C)

Figure 4. Photographs of ovarian tissue at room temperature in pomegranate flower extract ×50 (A) pH 1-2, (B) Without extract, (C) pH 4-5.

room temperature. In a short period of time, they achieved successful dyeing in dense concentration. In this study, we tried to stain both testis and ovarian tissues at two different temperatures and pH values. When the pH value of the paint extract was 4-5, there was a lighter color in the tissue at room temperature, and not pink. However, when the pH value of the dye extract is 1-2, the spermatogenic and interstitial cells were well defined as a purple-pink color. But this did not change the color of the ovarian tissue. This is thought to be due to the fact that the distribution and amount of molecules in the basic or acidic cells in both tissues are different. At 100°C dyes, even darker, brown dyes were observed in the section of the testis. This is the positive effect of temperature increase on dyeing yield.

The ovum showed diffuse cytoplasmic staining at different temperatures with different colors. The nucleus was unstained in tissues. Ovarian tissue was not affected by change in pH. This is seen in Figure 4a, c. Ovarian tissue was not dyed in Figure 4b. When compared with others, follicular and epithelial cells in testis tissue were stained (Figures 2s and 3). The most acidic dye solution gave positive dyeing result in testis tissue (Figures 5a and 3b). From this, testis tissue has more basic molecules or cells than ovarian tissue.

Our findings suggest that pomegranate flower or *P. granatum* flower can be mostly used as a stain for testis histological examination and histopathological diagnoses than the examination of ovarian tissue. But, hematoxylin is an acidic, plant-derived nucleus dye. In particular, the testis tissue was stained by pomegranate flower extract in strongly acidic medium having colour like hematoxylin. After working to increase the intensity of color, this extract will be nominated for hematoxylin equivalence. Because eosin is a synthetic dye, we do not use it to compare the color that the pomegranate flower extract



(A) **(B)** **(C)**

Figure 5. Photographs of testis tissue at room temperature in pomegranate flower extract ×50 (A) pH 1-2, (B) Without extract, (C) pH 4-5.

gives to testis and ovarian tissues. This work is the first study of the testis and ovary staining, and we hope to obtain better staining results with subsequent studies, to compare the results with hematoxylin control stains and show the results more photomicrographically.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

ACKNOWLEDGMENT

The author would like to thank Arzu Hanım Yay for his advice and suggestions given for this work.

REFERENCES

Avwioro OG (2002). Histochemistry and Tissue Pathology, 1st ed. Claverianun press Nigeria pp. 134-213.

Babu KD (2010). Floral Biology of Pomegranate (*Punica granatum* L.) Fruit, Vegetable and Cereal Science and Biotechnology. Global Science Books 4(2):51-55.

Bagri P, Ali M, Aeri V, Bhowmik M, Sultana S,(2009). Antidiabetic effect of *Punica granatum* flowers: effect on hyperlipidemia, pancreatic cells lipid peroxidation and antioxidant enzymes in experimental diabetes. Food and Chemical Toxicology 47(1): 50-54.

Bassey RB, Bakare AA, Peter Al, Oremosu AA, Osinubi AA (2012). Factors Influencing extract of *Hibiscus sabdariffa* Staining of Rat Testis. Biotechnic and Histochemistry 87(6):403-407.

Beigi Boroujeni M, Shahrokhi SS, Birjandi M, Abbaszadeh A, Beyranvand F, Hamoleh S, Zandbaf Z, Gholami M (2017). Effects of pomegranate peel extract on histopathology, testosterone levels and sperm of testicular torsion-detorsion induced in adult Wistar rats. Journal of Complemantary and Integrative Medicine 14(4):20170009.

Chandra R, Babu KD (2010). Propagation of Pomegranate- A Review. Fruit, Vegetable and Cereal Science and Biotechnology.Global Science Books 4(2):45-50.

Davulcu A, Benli H, SenY, Bahtiyari Mİ (2014). Dyeing of cotton with thyme and pomegranate peel.Cellulose 21:4671-4680.

Deng Y, Li Y, Yang F, Zeng A, YangS, LuoY, Zhang Y, Xie Y, Ye T, Xia Y, Yin W (2017). The extract from *Punica granatum* (pomegranate) peel induces apoptosis and impairs metastasis in prostate cancer cells. Biomedicine & Pharmacotherapy 93: 976-84.

Guler KN, Benli H (2017). Staining Effect of Pomegranate Flower Extract on Human Blood Cells: First Results. Journal of Environmental Science and Engineering A 6(2):249-251.

Hu YL, Luo, JY, Hu SR, Fu H, YangSH, Yang MH (2017) Application of natural plant pigments in enlarged health industry. U.S. National Library of Medicine 42(13):2433-2438.

Mahmoud AM, Nawwar Sahar AM, Hussein Irmgard M (1994). NMR Spectral analysis of polyphenols from *Punica granatum.* Phytochemistry 36(3):793-798.

Nagar Kh, El SanadSH, Mohomed AS, Ramadan A (2005). Mechanical Properties and Stability to Light Exposure for Dyed Egyptian Cotton Fabrics with Natural and Synthetic Dyes. Polymer-Plastics Technology and Engineering 44(7):1269-1279.

Pangello S, Destri Nicosia MG, Agosteo GE, Abdelfattah A, Romeo FV, Cacciola SO, Rapisarda P, Schena L (2017). Evalution of a Pomegranate Peel Extract as Alternative Mean to Control Olive Anthracrose. Phtopathology 107(12):1462-1467

Wang R, Ding Y, LiuR, Xiang L, Du L (2010). Pomegranate: Constituents, Bioactivities and Pharmacokinetics. Fruit, Vegetable and Cereal Science and Biotechnology. Global Science Books 4(2):77-87.

Wang R, Wang W, Wang L, Liu R, Ding Y, Du L (2006). Contituents of the flowers of *Punica granatum.* Fitoterapia 77:534-537.

Xu J, Zhao Y, Aisa HA (2017). Anti-inflamatory effect of pomegranate flower in lipopolysacchoride stimulated RAW 264. 7 macrophages. Pharmaceutical Biology 55(1):2095-2101.

Zeliha S, Havva Eda U,Senay O (2016). Role of Aromatic Plants on Traditional and Complementary Alternative Medicine Practises. Research & Reviews: Research Journal of Biology 4(2:52-3.

Zollinger H (2004). Color Chemistry. Syhtheses, Properties, and Applications of Organic Dyes and Pigments, 3rd ed. John Wiley and Sons. New York. pp. 544-545.

Vol. 17(32), pp. 994-1000, 8 August, 2018
DOI: 10.5897/AJB2018.16471
Article Number: 9E8BCC658072
ISSN: 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Circular RNA expression profiles of peripheral blood mononuclear cells in rheumatoid arthritis patients

Xinqiang Song[1,2*], Erqin Dai[1], Pengpeng Zhang[1], Shuai Wang[1], Lei Wang[1], Lei Chen[1], Yapeng Han[1] and Yuan Luo[3#]

[1]College of Life Sciences, Xinyang Normal University, Xinyang, China.
[2]Institute for Conservation and Utilization of Agro-Bioresources in Dabie Mountains, China.
[3]State Key Laboratory of Toxicology and Medical Countermeasures, Institutes of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing, China.

Received 30 March, 2018; Accepted 20 July, 2018

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disease involving synovial inflammation and joint destruction. Lack of knowledge on RA pathogenesis hinders development of effective treatments. The aim of this study was to examine whether circular RNAs (circRNAs), which are involved in the development of other diseases, may also play a role in RA. Expression profiles of circRNAs were compared between peripheral blood lymphocytes from RA patients and healthy controls. Candidate RA-associated circRNAs were validated using RT-PCR, and interactions with target microRNAs were predicted. Analysis of circRNA microarrays identified 689 circRNAs expressed at significantly different levels between RA patients and controls ($p < 0.05$), of which 95 were up-regulated and 594 down-regulated in RA. Microarray analysis was corroborated by RT-PCR for hsa_circRNAs 103571, 101319, 102034, 103503 and 100257. Potential target miRNAs for hsa_circRNAs were predicted. Several circRNAs in peripheral blood lymphocytes may be differentially expressed in RA (hsa_circRNAs 103571, 101319, 102034, 103503 and 100257). These circRNAs may serve as potential targets for RA diagnosis and treatment.

Key words: Circular RNA, microarray, rheumatoid arthritis, microRNA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease involving synovial inflammation and joint destruction that affects approximately 1% of the general population. It is widely accepted that the pathogenesis of RA is characterized by systemicinflammation and autoimmunity with multiple adjacent cartilage and bone lesions (McInnes and Schett, 2011). RA is a chronic disease with severe complications, whichresults in short- or long-term disability and increased mortality. However, the etiology and pathogenesis of RA remain largely unknown, which hinders efforts to develop therapeutic strategies.

Both genetic and environmental factors are thought to play a role in disease development and disease

*Corresponding author. E-mail: xqsong2012@126.com.

#Authors contributed equally

progression. Efforts to identify genetic factors of RA have focused on HLA, PTPN22 and CTLA4, with over 100 genetic risk loci described so far (Wellcome Trust Case Control, 2007). Each risk locus harbors multiple candidate genes and whether any of these genes actually cause the disease is unknown.

Circular RNAs (circRNAs), non-coding RNAs of which more than 25,000 exist in various human cell types (Chen et al., 2015; Jeck et al., 2013), have been implicated in the development of several types of diseases, including atherosclerosis and nervous system disorders (Burd et al., 2010; Chen et al., 1990; You et al., 2015). These RNAs undergo splicing even though they lack a free 3′ or 5′ end (Hentze and Preiss, 2013; Vicens and Westhof, 2014), and they are expressed in certain tissues at certain developmental stages (Memczak et al., 2013; Salzman et al., 2013). Though circRNAs has been measured in tissue, serum, exosomes and other body fluids in several kinds diseases (Kleaveland et al., 2018; Kristensen et al., 2018; Zhou et al., 2018), few studies have examined whether circRNAs may play a role in RNA.

To address this question, the present study used circRNA microarrays to compare expression profiles in peripheral blood lymphocytes between RA patients and healthy controls. The resulting set of differentially expressed circRNAs was analyzed using bioinformatics to predict potential target miRNAs. These results may help identify circRNAs that can serve as RA biomarkers, and their further study may help elucidate pathways driving RA pathogenesis.

MATERIALS AND METHODS

Three RA patients (1 man and 2 women; age range of 50 to 62 years) were recruited from outpatients at the Department of Physical Medicine in the First Affiliated People's Hospital of Xinyang. The patients were diagnosed of RA based on American College of Rheumatology criteria. As controls, three healthy volunteers were recruited (1 man and 2 women; age range of 49 to 63 years).

None of the subjects in this study had diabetes, malignancy or diseases of the kidney or liver, any of which can influence the circRNA expression profile. The study protocol was approved by the Ethics Committee of Xinyang Normal University, and informed consent was obtained from all subjects.

Analysis of circRNA microarrays

Peripheral blood lymphocytes were isolated from RA patients and controls as described (Viecceli et al., 2017). RNA from each sample was subjected to microarray analysis and hybridization according to the manufacturer's protocol (Arraystar, MD, USA). In brief, total RNA was digested with RNaseR (Epicentre, CA, USA) to remove linear RNAs, and the enriched circular RNAs were amplified and transcribed into fluorescent cRNA using a random priming method (Super RNA Labeling Kit, Arraystar). The labeled cRNAs were purified using the RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using the NanoDrop ND-1000. Labeled cRNA (1 μg) was fragmented by adding 5 μl 10 × Blocking Agent and 1 μl 25 × Fragmentation Buffer, the mixture was heated at 60°C for 30 min, and finally the labeled cRNA was diluted with 25 μl of 2 × hybridization buffer. Hybridization solution (50 μl) was dispensed into the gasket slide, which was assembled with the circRNA expression microarray slide. Slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven (G2545A). The hybridized arrays were washed, fixed and scanned using the Agilent Scanner G2505C; array images were analyzed using Agilent Feature Extraction software (version 11.0.1.1). Normalization and subsequent data processing were performed using the R software package. Volcano plot filtering was used to identify circRNAs that differed significantly between patient and control samples. Hierarchical clustering was used to generate circRNA expression profiles for the two groups and to compare them.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was reverse-transcribed into cDNA in reactions containing 1.0 μg of purified total RNA, 4.0 μl of reaction buffer, 0.5 μl of random primers, 0.5 μl of oligo(dT), 2.0 mM dNTP, 0.5 μl of reverse transcriptase and 0.5 μl of RNase inhibitor. Quantitative RT-PCR was performed using the ABI 7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA), SYBR Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) and the primers shown in Table 1. Three independent samples were analyzed in triplicate for RA patients and controls, with β-actin as internal control. Primer specificity was confirmed by melt curve analysis after the PCRs. Relative expression of circRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method.

Bioinformatics analysis

Interactions between circRNAs and potential target miRNAs were predicted using Arraystar miRNA target prediction software, which is based on TargetScan (www.targetscan.org) and miRanda. This analysis was used to identify the five most likely target miRNAs, whose putative target genes were identified by Targetscan.

Statistical analysis

All experiments were performed and analyzed in triplicate. Results are reported as mean ± standard deviation (SD). Differences between patients and controls were assessed for significance using Student's *t* test, and a threshold of $P < 0.05$ was defined for statistical significance. Data were analyzed using SPSS 23 (IBM, Chicago, IL, USA).

RESULTS

circRNA expression profiles of peripheral blood lymphocytes

Hierarchical clustering was used to generate circRNA expression profiles for RA samples and control samples (Figure 1A), and scatter and volcano plots revealed differences between the circRNA expression profiles

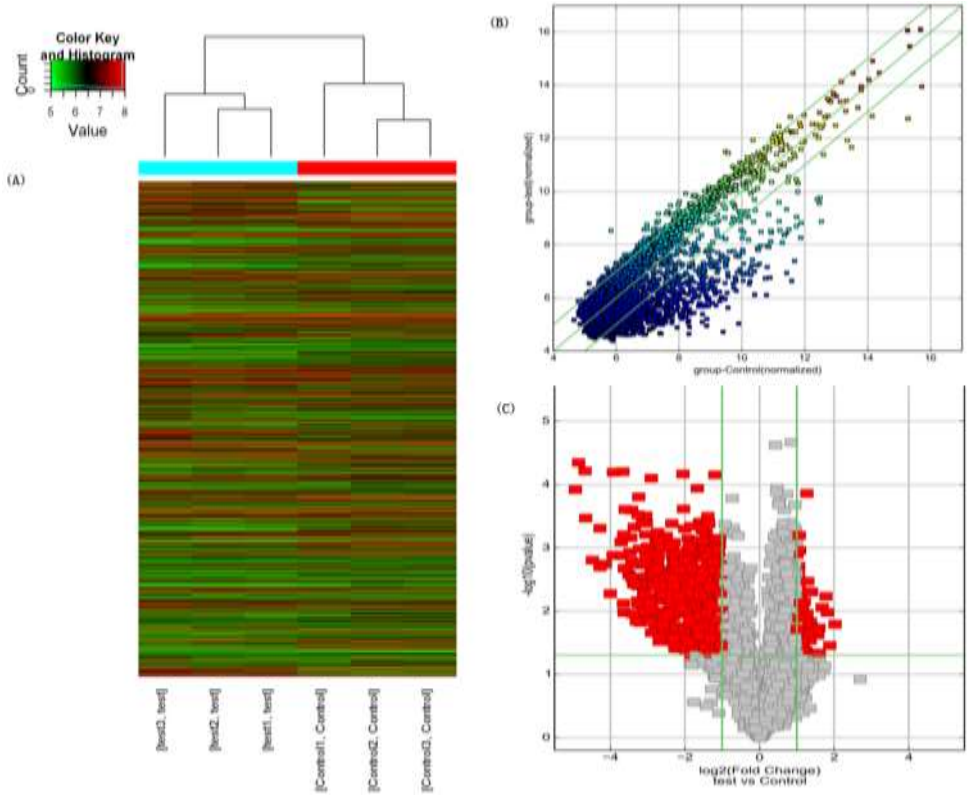


Figure 1. Differential expression of circRNAs in peripheral blood lymphocytes from RA patients and healthy controls. (A) Hierarchical clustering analysis, in which each column represents one sample and each row represents one circRNA. Data were analyzed from three patients and three controls. Red color indicates circRNAs up-regulated in RA; green, circRNAs down-regulated in RA. (B) Scatter plot showing variation in circRNA expression between control samples (normal) and RA samples (test). Values on the *x*- and *y*-axes correspond to the normalized signals (log_2 scaled). Green lines indicate fold-changes; circRNAs above the top green line and below the bottom green line vary more than 2.0-fold between control and RA samples. (C) Volcano plots were constructed based on fold-change values and *p* values. The vertical lines correspond to up- and down-regulation by 2.0-fold between control and RA (test) samples. The horizontal line represents a *p* value. Red points represent circRNAs expressed at significantly different levels between controls and patients.

Table 1. Specific circRNA primers used for quantitative RT-PCR analysis.

Target	Primer sequence	Product size (bp)
β-actin (Human)	F: 5' GTGGCCGAGGACTTTGATTG3' R: 5' CCTGTAACAACGCATCTCATATT3'	73
hsa_circRNA_103571	F: 5'AACTGACGGTGTTGTTCTTTG3' R: 5'AACTGTTTCTGTTGCAGGTGA3'	136
hsa_circRNA_101319	F: 5'CAGCAACAGTGGCAATGAGA3' R: 5'TTATAGGGAGCTTCCAGCATG3'	120
hsa_circRNA_102034	F: 5'GTCATCTGTATAGTGTATGCCGTTA3' R: 5'CAGCTGGAATGGTGATTTCTT3'	138
hsa_circRNA_103503	F: 5'AGCCTGGAATCACGAAGCACA3' R: 5'TGACGATGACGACGAGACAACA3'	136
hsa_circRNA_100257	F: 5'CCTAAGTCTTATCGAACTGCCA3' R: 5'GATGTGTCTTTGAGGGTGTCTTT3'	160

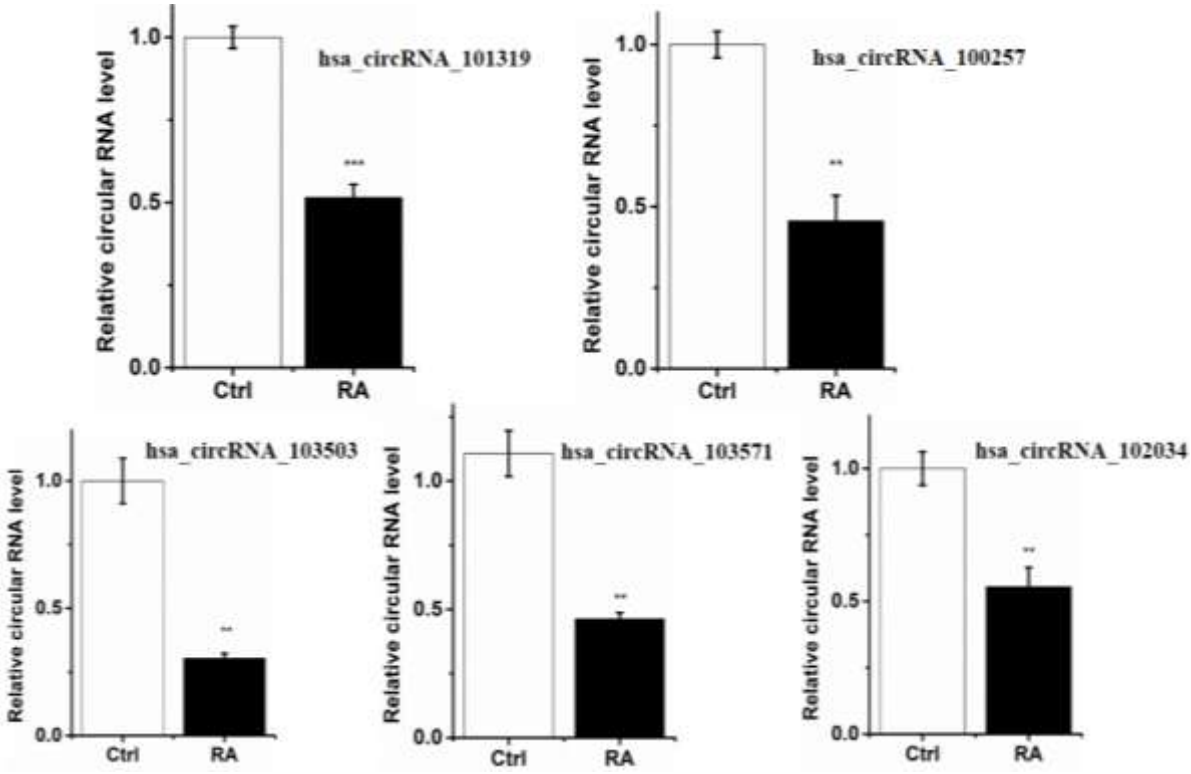


Figure 2. RT-PCR validation of a subset of candidate differentially expressed circRNAs in RA. Data shown are mean ± SD; ** $p < 0.01$, *** $p < 0.05$.

(Figure 1B and C). A total of 689 circRNAs were differentially expressed: 95 were up-regulated in RA, while 594 were down-regulated (Table 1).

Validation of candidate circRNAs using qRT-PCR

The candidate RA-associated hsa_circRNAs 103571, 101319, 102034, 103503 and 100257 were selected and their differential expression was validated using quantitative RT-PCR. The levels of all circRNAs were found to be significantly lower in RA patients (Figure 2), consistent with the microarray data.

Prediction and annotation of circRNA-miRNA networks

Targetscan and miRanda were used to predict miRNAs targeted by the circRNAs differentially expressed in RA. Table 2 shows the top 10 up- and down-regulated circRNAs, together with their predicted miRNA targets. Differentially expressed circRNAs were annotated in terms of their genes (expressing the circRNA or near the genes expressing the circRNA) and miRNA response elements (Figure 3).

DISCUSSION

Many RA studies have focused on the epigenetic regulation of its pathogenesis and potential targets for therapy, including miRNAs and long noncoding RNAs (lncRNAs). In this study, circRNA expression in peripheral blood lymphocytes between RA patients and controls were compared and 689 circRNAs significantly altered in RA patients were identified, out of which, 95 were up-regulated and 594 down-regulated in the disease. However, the occurrence of circRNAs remains largely unknown.

The results show several circRNAs that may be useful as biomarkers or targets in RA. These findings expand the list of diseases in which circRNAs have been implicated (Ghosal et al., 2013; Glazar et al., 2014; Li et al., 2014; Zheng et al., 2016), which include Parkinson's and Alzheimer's diseases (Lin et al., 2016; Lukiw, 2013; Rybak-Wolf et al., 2015), colorectal cancer (Bachmayr-Heyda et al., 2015), oesophageal squamous cell carcinoma (Li et al., 2015a), gastric cancer (Li et al., 2015b), hepatocellular carcinoma (Qin et al., 2016), atherosclerotic vascular disease (Burd et al., 2010), myotonic dystrophy (Ashwal-Fluss et al., 2014) and CD28-dependent CD8(+) T cell ageing (Wang et al., 2015).

Table 2. Characteristics and predicted miRNA targets of the top 10 up- and down-regulated circRNAs in RA*.

P-value	FC (abs)	Level in RA (*vs* healthy)	circRNA	Alias	GeneSymbol	MRE1	MRE2	MRE3	MRE4
0.000119396	30.7312059	down	hsa_circRNA_103503	hsa_circ_0001965	PHC3	hsa-miR-582-3p	hsa-miR-542-3p	hsa-miR-655-3p	hsa-miR-206
4.46066E-05	28.9573651	down	hsa_circRNA_101835	hsa_circ_0005615	NFATC3	hsa-miR-744-5p	hsa-miR-661	hsa-miR-9-5p	hsa-miR-377-5p
6.07889E-05	25.6015442	down	hsa_circRNA_101836	hsa_circ_0000711	NFATC3	hsa-miR-744-5p	hsa-miR-576-3p	hsa-miR-661	hsa-miR-9-5p
0.000338131	25.4155416	down	hsa_circRNA_101550	hsa_circ_0035796	HERC1	hsa-miR-877-3p	hsa-miR-130b-5p	hsa-miR-18a-3p	hsa-miR-26b-3p
0.001560238	22.3442666	down	hsa_circRNA_104052	hsa_circ_0008285	CDYL	hsa-miR-892a	hsa-miR-378a-5p	hsa-miR-887-5p	hsa-miR-22-5p
0.000490694	19.4689185	down	hsa_circRNA_102927	hsa_circ_0058493	RHBDD1	hsa-miR-182-5p	hsa-miR-196a-3p	hsa-miR-28-5p	hsa-miR-330-3p
0.002004659	19.396182	down	hsa_circRNA_103572	hsa_circ_0008351	LRCH3	hsa-miR-29b-2-5p	hsa-miR-544a	hsa-miR-29a-5p	hsa-miR-518c-5p
0.001809156	17.578193	down	hsa_circRNA_101237	hsa_circ_0003489	CDK8	hsa-let-7c-5p	hsa-miR-98-5p	hsa-let-7a-5p	hsa-let-7b-5p
0.005274305	16.0433786	down	hsa_circRNA_101231	hsa_circ_0000467	SKA3	hsa-miR-153-5p	hsa-miR-382-5p	hsa-miR-520g-3p	hsa-miR-549a
0.00130994	15.1884894	down	hsa_circRNA_100913	hsa_circ_0002884	PICALM	hsa-miR-656-5p	hsa-miR-361-3p	hsa-miR-421	hsa-miR-563
0.026636248	2.4575359	up	hsa_circRNA_000046	hsa_circ_0000059	CAP1	hsa-miR-21-3p	hsa-miR-637	hsa-miR-128-2-5p	hsa-miR-296-5p
0.000137307	2.410555	up	hsa_circRNA_000442	hsa_circ_0001625	BACH2	hsa-miR-580-5p	hsa-miR-139-3p	hsa-miR-877-3p	hsa-miR-603
0.016132485	4.0707993	up	hsa_circRNA_000684	hsa_circ_0001013	KIAA1841	hsa-miR-30d-3p	hsa-miR-185-5p	hsa-miR-30e-3p	hsa-let-7b-5p
0.017315747	2.0142591	up	hsa_circRNA_000776	hsa_circ_0001929	XLOC	hsa-miR-128-1-5p	hsa-miR-128-2-5p	hsa-miR-146a-3p	hsa-miR-93-3p
0.0463824	2.0501922	up	hsa_circRNA_001012	hsa_circ_0000254	RRP12	hsa-miR-125b-5p	hsa-miR-125a-5p	hsa-miR-184	hsa-miR-1224-5p
0.004958353	2.6277342	up	hsa_circRNA_001241	hsa_circ_0000508	CUL4A	hsa-miR-342-3p	hsa-miR-449b-3p	hsa-miR-377-3p	hsa-miR-432-3p
0.002992558	2.0532735	up	hsa_circRNA_001754	hsa_circ_0000559	FOXN3	hsa-miR-136-5p	hsa-miR-877-3p	hsa-miR-421	hsa-miR-153-3p
0.015774197	2.1293544	up	hsa_circRNA_001820	hsa_circ_0000728	MC1R	hsa-miR-1296-5p	hsa-miR-770-5p	hsa-miR-384	
0.023862644	2.9435674	up	hsa_circRNA_002039	hsa_circ_0001748	CHCHD3	hsa-miR-624-5p	hsa-miR-28-5p	hsa-miR-511-3p	hsa-miR-545-5p
0.022712291	2.0650012	up	hsa_circRNA_002042	hsa_circ_0000204	WDR37	hsa-miR-1-3p	hsa-miR-22-5p	hsa-miR-656-3p	hsa-miR-302c-5p

FC (abs), fold-change; MRE, miRNA response element; circRNAs are sorted by their fold-change. MREs were predicted using miRNA target prediction software (Arraystar).

How circRNAs function in health and disease is unclear. Evidence is growing that they act as molecular sponges to "mop up" target miRNAs and thereby regulate the ability of miRNAs to inhibit gene expression. For example, the circRNA ciRS-7 contains miRNA-7 binding sites, which allow it to bind miRNA-7 and regulate gene expression in Parkinson's and Alzheimer's diseases (Lin et al., 2016; Lukiw, 2013; Rybak-Wolf et al., 2015). Some of the differentially expressed circRNAs identified in the present study are predicted to bind miRNA targets that have previously been linked to RA. Several studies (Churov et al., 2015; Pauley et al., 2008) have reported up-regulation of hsa-miR-16-5p in peripheral blood mononuclear cells, plasma and synovial fluid of RA patients; miR-16 targets the 3′-UTR of tumor necrosis factor-α, a key pro-inflammatory mediator in RA (Ouboussad et al., 2017; Sujitha and Rasool, 2017). The miRNAs miR-24-3p and miR125a-5p stimulate production of chemokine and inflammatory cytokines and may be helpful in RA diagnosis (Hong et al., 2017; Lai et al., 2017). Many of the predicted miRNA binding sites in the circRNAs identified in the present study are likely to be functional (Hansen et al., 2013; Liu et al., 2017; Migita et al., 2017; Zhang et al., 2017). Future studies should validate the miRNA binding predictions here and exploit these circRNA-miRNA interactions to elucidate the pathogenesis of RA.

CircRNAs can be categorized into three types: exonic, intronic and exonic-intronic. Exonic circRNAs are large molecules comprising exons and are considered by-products of exon skipping, either in pre-messenger RNA (mRNA)

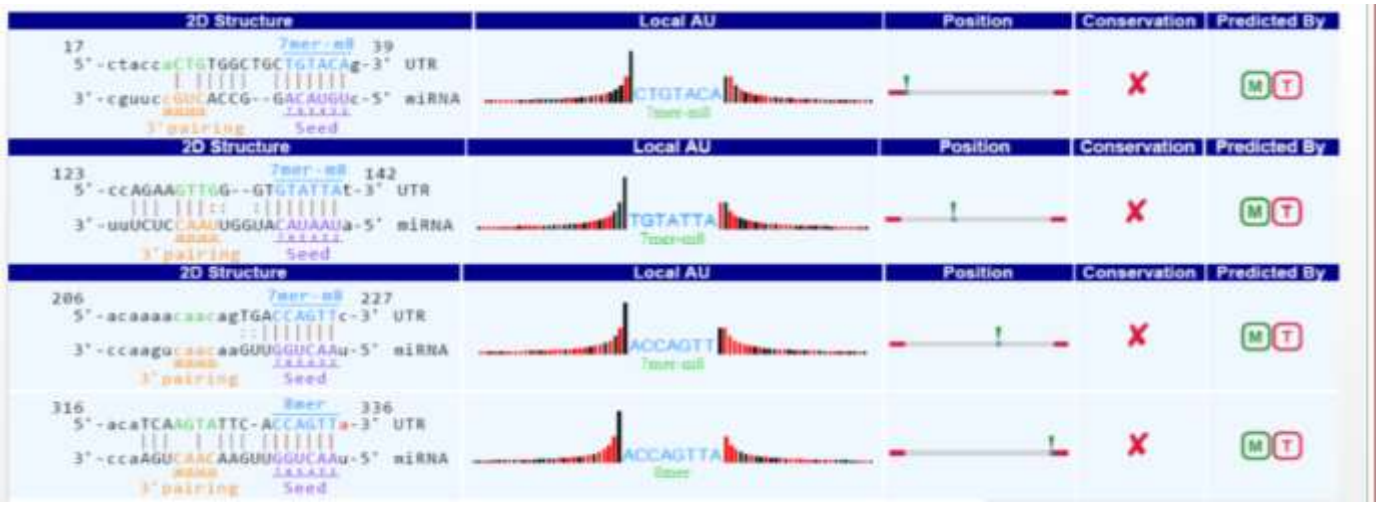


Figure 3. Annotation of predicted circRNA−miRNA interactions. Example of annotation results showing complementarity of hsa_circRNA_103503 with hsa-miR-582-3p, hsa-miR-542-3p and hsa-miR-655-3p.

splicing or in mature mRNA re-splicing (Chen et al., 2016; Wang, 2015). Intronic circRNAs are produced by joining two or more introns, which is rare in eukaryotic cells. Exonic-intronic circRNAs are enriched at transcription sites and may promote transcription of the corresponding parental mRNAs. Recent studies have shown that many exonic transcripts can form circRNAs through non-linear reverse splicing or gene rearrangement. Both exonic and intronic circRNAs may help regulate gene expression (Ebbesen et al., 2016; Li et al., 2015c). In general, circRNAs are more highly conserved and stable than miRNAs and lncRNAs, (Hansen et al., 2013) which makes circRNAs particularly attractive as biomarkers and therapeutic targets (Li et al., 2015b) The present work identifies several circRNAs differentially expressed in RA, and these molecules may help drive the disease by binding miRNAs to regulate gene expression. Future work should examine circRNA-miRNA interactions and the genes affected in order to gain insights into RA pathogenesis as well as explore potential diagnostic tools and therapeutic targets.

CONFLICTS OF INTEREST

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work was funded by the Henan Science and Technology Innovation Team, Investigation on Plant Resources in Dabie Mountains and the Study and Utilization of Active Components of Special Plants, the Nanhu Scholars Program of Young Scholars of Xinyang Normal University, and the College Students' Scientific Research Project (2014).

ABBREVIATIONS

RNA, Ribonucleic acid; RA, rheumatoid arthritis; RT-PCR, reverse transcription-polymerase chain reaction; miRNA, microRNA; lncRNAs, long noncoding RNAs.

REFERENCES

Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O (2014). circRNA biogenesis competes with pre-mRNA splicing. Molecular Cell 56(1):55-66. doi:10.1016/j.molcel.2014.08.019

Bachmayr-Heyda A, Reiner AT, Auer K, Sukhbaatar N, Aust S, Bachleitner-Hofmann T, Mesteri I, Grunt TW (2015). Correlation of circular RNA abundance with proliferation--exemplified with colorectal and ovarian cancer, idiopathic lung fibrosis, and normal human tissues. Scientific Reports 5:8057 doi:10.1038/srep08057.

Burd CE, Jeck WR, Liu Y, Sanoff HK, Wang Z, Sharpless NE (2010). Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. PLoS Genetics 6(12):1001233. doi:10.1371/journal.pgen.1001233.

Chen L, Huang C, Wang X, Shan G (2015). Circular RNAs in Eukaryotic Cells. Current genomics 16(5):312-318. doi:10.2174/1389202916666150707161554.

Chen Y, Li C, Tan C, Liu X (2016). Circular RNAs: a new frontier in the study of human diseases. Journal of Medical Genetics doi:10.1136/jmedgenet-2016-103758.

Chen YT, Li C, Tan C (1990). Cerebellar degeneration-related antigen: a highly conserved neuroectodermal marker mapped to chromosomes X in human and mouse. Proceedings of the National Academy of Sciences of the United States of America 87(8):3077-3081.

Churov AV, Oleinik EK, Knip M (2015). MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. Autoimmunity Reviews 14(11):1029-1037. doi:10.1016/j.autrev.2015.07.005.

Ebbesen KK, Kjems J, Hansen TB (2016). Circular RNAs: Identification, biogenesis and function. Biochimica et Biophysica Acta 1859(1):163-168. doi:10.1016/j.bbagrm.2015.07.007.

Ghosal S, Das S, Sen R, Basak P, Chakrabarti J (2013). Circ2Traits: a comprehensive database for circular RNA potentially associated with disease and traits. Frontiers in Genetics 4:283 doi:10.3389/fgene.2013.00283.

Glazar P, Papavasileiou P, Rajewsky N (2014). circBase: a database for circular RNAs. Rna 20(11):1666-1670. doi:10.1261/rna.043687.113.

Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J (2013). Natural RNA circles function as efficient microRNA sponges. Nature 495(7441):384-388.doi:10.1038/nature11993

Hentze MW, Preiss T (2013) Circular RNAs: splicing's enigma variations. The EMBO journal 32(7):923-925 doi:10.1038/emboj.2013.53

Hong BK, Pengying Z, Xinming W, Jiajie T, Wei W (2017). MicroRNA-143 and -145 modulate the phenotype of synovial fibroblasts in rheumatoid arthritis. Experimental Molecular Medicine 49(8):363. doi:10.1038/emm.2017.108

Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, Marzluff WF, Sharpless NE (2013). Circular RNAs are abundant, conserved, and associated with ALU Repeats. Rna 19(2):141-157 doi:10.1261/rna.035667.112

Kleaveland B, Shi CY, Stefano J, Bartel DP (2018). A Network of Noncoding Regulatory RNAs Acts in the Mammalian Brain. Cell doi:10.1016/j.cell.2018.05.022

Kristensen LS, Okholm TLH, Veno MT, Kjems J (2018). Circular RNAs are abundantly expressed and upregulated during human epidermal stem cell differentiation. RNA Biol 15(2):280-291 doi:10.1080/15476286.2017.1409931

Lai NS, Yu HC, Tung CH, Huang KY, Huang HB, Lu MC (2017). The role of aberrant expression of T cell miRNAs affected by TNF-alpha in the immunopathogenesis of rheumatoid arthritis. Arthritis research and therapy 19(1):261 doi:10.1186/s13075-017-1465-z

Li F, Zhang L, Li W, Deng J, Zheng J, An M, Lu J, Zhou Y (2015a). Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/beta-catenin pathway. Oncotarget 6(8):6001-6013 doi:10.18632/oncotarget.3469

Li JH, Liu S, Zhou H, Qu LH, Yang JH (2014). starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic acids research 42(Database issue):D92-7 doi:10.1093/nar/gkt1248

Li P, Chen S, Chen H, Mo X1, Li T, Shao Y, Xiao B (2015b). Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clinica chimica acta; international Journal of Clinical Chemistry 444:132-136 doi:10.1016/j.cca.2015.02.018

Li Z, Huang C, Bao C, Chen L, Lin M, Wang X, Zhong G, Yu B, Hu W, Dai L, Zhu P (2015c). Exon-intron circular RNAs regulate transcription in the nucleus. Nature Structural Molecular Biology 22(3):256-264 doi:10.1038/nsmb.2959

Lin SP, Ye S, Long Y, Fan Y, Mao HF, Chen MT, Ma QJ (2016). Circular RNA expression alterations are involved in OGD/R-induced neuron injury. Biochemical and Biophysical Research Communications 471(1):52-56 doi:10.1016/j.bbrc.2016.01.183

Liu J, Fei D, Xing J, Du J (2017). MicroRNA-29a inhibits proliferation and induces apoptosis in rheumatoid arthritis fibroblast-like synoviocytes by repressing STAT3. Biomed Pharmacother 96:173-181 doi:10.1016/j.biopha.2017.09.120

Lukiw WJ (2013). Circular RNA (circRNA) in Alzheimer's disease (AD). Frontiers in Genetics 4:307 doi:10.3389/fgene.2013.00307

McInnes IB, Schett G (2011). The pathogenesis of rheumatoid arthritis. The New England Journal of Medicine 365(23):2205-2219. doi:10.1056/NEJMra1004965

Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 495(7441):333-338. doi:10.1038/nature11928.

Migita K, Nozomi I, Yasumori I, Kawahara C (2017). TNF-alpha-induced miR-155 regulates IL-6 signaling in rheumatoid synovial fibroblasts. BMC Research Notes 10(1):403 doi:10.1186/s13104-017-2715-5.

Ouboussad L, Hunt L, Hensor EM, Nam JL, Barnes NA, Emery P, McDermott MF, Buch MH (2017). Profiling microRNAs in individuals at risk of progression to rheumatoid arthritis. Arthritis Research Therapy 19(1):288 doi:10.1186/s13075-017-1492-9

Pauley KM, Satoh M, Chan AL, Bubb MR, Reeves WH, Chan EK (2008). Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. Arthritis Research Therapy 10(4):101. doi:10.1186/ar2493.

Qin M, Gangc L, Xisongb H, Xuemeib T Xiaomenga S (2016). Hsa_circ_0001649: A circular RNA and potential novel biomarker for hepatocellular carcinoma. Cancer biomarkers: section A of Disease Markers 16(1):161-169. doi:10.3233/CBM-150552.

Rybak-Wolf A, Stottmeister C, Glažar P, Jens M, Pino N (2015). Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed. Molecular Cell 58(5):870-885. doi:10.1016/j.molcel.2015.03.027.

Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO (2013). Cell-type specific features of circular RNA expression. PLoS Genetics 9(9):1003777. doi:10.1371/journal.pgen.1003777.

Sujitha S, Rasool M (2017). MicroRNAs and bioactive compounds on TLR/MAPK signaling in rheumatoid arthritis. Clinica Chimica Acta 473:106-115. doi:10.1016/j.cca.2017.08.021.

Vicens Q, Westhof E (2014). Biogenesis of Circular RNAs. Cell 159(1):13-4 doi:10.1016/j.cell.2014.09.005

Viecceli D, Mariana PG, Laiana S, Ana Paula A (2017) .Correlation between cellular expression of complement regulatory proteins with depletion and repopulation of B-lymphocytes in peripheral blood of patients with rheumatoid arthritis treated with rituximab. Revista Brasileira de Reumatologia Engl 57(5):385-391. doi:10.1016/j.rbre.2016.09.007.

Wang YH, Yu XH, Luo SS, Han H (2015). Comprehensive circular RNA profiling reveals that circular RNA100783 is involved in chronic CD28-associated CD8(+)T cell ageing. Immunity Ageing 1:12-17. doi:10.1186/s12979-015-0042-z.

Wang Z (2015) Not just a sponge: new functions of circular RNAs discovered. Science China Life Sciences 58(4):407-408. doi:10.1007/s11427-015-4826-3.

Wellcome Trust Case Control C (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447(7145):661-678. doi:10.1038/nature05911.

You X, Vlatkovic I, Babic A, Will T (2015). Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. Nature Neuroscience 18(4):603-610. doi:10.1038/nn.3975

Zhang C, Peng L, Zhang Y, Liu Z, Li W, Chen S, Li G (2017). The identification of key genes and pathways in hepatocellular carcinoma by bioinformatics analysis of high-throughput data. Medical Oncology 34(6):101. doi:10.1007/s12032-017-0963-9.

Zheng LL, Li JH, Wu J, Sun WJ, Liu S, Wang ZL, Zhou H, Yang JH, Qu LH (2016). deepBase v2.0: identification, expression, evolution and function of small RNAs, LncRNAs and circular RNAs from deep-sequencing data. Nucleic Acids Research 44(1):196-202 doi:10.1093/nar/gkv1273.

Zhou TC, Li X, Chen LJ, Fan JH, Lai X, Tang Y, Zhang L, Wei J (2018). Differential expression profile of hepatic circular RNAs in chronic hepatitis B. Journal of Viral Hepatitis doi:10.1111/jvh.12944 https://www.ncbi.nlm.nih.gov/pubmed/29888838.

Vol. 17(32), pp. 1001-1006, 8 August, 2018
DOI: 10.5897/AJB2018.16552
Article Number: 5FD56C758076
ISSN: 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Comparative analysis of the chemical compositions of indigenous watermelon (*Citrullus lanatus*) seeds from two districts in Limpopo Province, South Africa

Emmanuel Alpheus Mogotlane, Phatlane William Mokwala and Phetole Mangena*

Department of Biodiversity, School of Molecular and Life Sciences, Faculty of Science and Agriculture, University of Limpopo, Private Bag X 1106, Sovenga 0727, South Africa.

Received 13 June, 2018; Accepted 16 July, 2018

Seeds from different indigenous watermelons were analysed for their chemical composition such as phenolics, proteins, oils, minerals, ash and fibre content as well as antioxidant activity measured in methanol extract using 2.2-diphenyl-1-picrylhydrazyl (DPPH) assay. The morphological variations of watermelons, which revealed diversity in terms of fruit and seed colour were also assessed. Average percentages for lipid content, total proteins, crude fibre, ash and total carbohydrates as 34.4, 16.5, 23.1, 3.99, and 3.16%, respectively, were recorded for Capricorn district and 31.6, 14.9, 22.0, 4.58, and 5.26% were recorded for Sekhukhune landraces. The Capricorn landraces had higher content of flavonoids (0.222 mg/g) than Sekhukhune varieties (0.130 mg/g). But, landraces from Sekhukhune had higher antioxidant activity (46.5%) which corresponded to higher total phenolic content of 0.59 mg/g. Thus, suggest that watermelon seeds may serve as potential source of natural products for food, feed and pharmaceutical applications.

Key words: Amino acids, *Citrullus lanatus*, flavonoids, phenolics, seeds, watermelon.

INTRODUCTION

Citrullus lanatus (watermelon) belongs to the family Cucurbitaceae, also referred to as cucurbits from a large group with approximately 800 species and 130 genera (Najafi et al., 2010). This family includes species like pumpkins, melons, gourds and squashes, that are cultivated worldwide for medicinal and consumption purposes. Plant breeding programmes in watermelons have been essential to develop new varieties of high quality fruit traits, productivity and resistance to biotic and abiotic stress factors. Watermelons have been found to be nutritionally rich due to the ß-carotene and vitamins B, C and E and demonstrated to contain significantly large amounts of lycopene (Choudhury et al., 2015). The phytochemical compounds found in watermelons such as phenolics, tannnins, flavonoids and carotenoids, particularly in seeds have gained interest among

*Corresponding author. E-mail: Phetole.Mangena@ul.ac.za.

consumers and scientists (Devasagayam et al., 2004; Addo et al., 2018). Watermelons, including plants such as gooseberries (Chiang et al., 2013), proved to contain large amounts of these phytochemicals and therefore, a good source of antioxidants. Red-flashed genotypes of watermelons were also reported by Choudhury et al. (2015) to contain variably high amounts and different phytochemicals and antioxidants. Among the major sources of phytochemicals, green leafy vegetables and grains are also rich sources of antioxidants apart from being high in energy, protein, essential oils and micronutrients. Furthermore, reports by Oyeleke et al. (2012) and Rahman et al. (2013) indicated that watermelons offer important nutritional and health-promoting compounds, like lycopene. Lycopene, predominantly found in red watermelon and tomato is a carotenoid with strong antioxidant capacity and potential health benefits (Edwards et al., 2003). Watermelon seeds contain high amounts of fatty acids, proteins and vitamins including copper and zinc. Mariod et al. (2009) suggested that watermelons have potential economic benefits especially due to chemicals contained in their seeds. The seeds can be used to prepare a number of consumables including snacks, flour and sauces (Tabiri et al., 2016). However, seeds are often discarded while only the fruit is eaten. The excellent nutritional properties in watermelon seeds have been reported (Badlfu, 1991). In this study, chemical compositions and antioxidant properties in seeds of watermelon fruits collected from two regions (Capricorn and Sekhukhune district) in the Limpopo province, South Africa are reported.

MATERIALS AND METHODS

Plant

C. lanatus landrace fruits used in this study were collected from Sekhukhune (01SDPW, 02SDPW, 03SDPW, and 05SDPW) and Capricorn (06CDGM, 07CDGM, 08CDGM, 09CDGM and 10CDGM) districts during harvesting period in autumn. The districts are situated in the centre of Limpopo province, classified as semi-arid areas, characterised by summer rainfalls and susceptible to frequent droughts. A total of 12 ripe watermelon fruits were randomly collected per landrace, properly washed and sliced to remove the seeds. The seeds were rinsed with dH_2O and air dried by spreading on clean paper towel in an aerated room for 12 h under room temperature. Seeds were randomly separated into 100 seeds samples, weighed and stored under dry conditions until they were analysed.

Seed oil extraction

For seed oil analysis, 10 g of ground seed powder was extracted using n-hexane in a Soxhlet apparatus for 24 h. Crude oil was recovered by evaporating the solvent under a draught of air. Determination of saponification and iodine values was performed according to IAFMM (1981) and George et al. (2013). The values were calculated using the formula: $56.1 (V_2 - V_1) / W$, where V_2 is the volume of HCl used in blank, V_1 is the volume of HCl used in sample and W is the weight of oil.

Estimation of seed protein by Bradford method

Crude proteins and amino acids were analysed as described by the American Association of Chemists (AOAC, 2006). Total proteins were determined from seed powder by colorimetric assay using bovine serum albumin (BSA) as a standard. A solution of 50 µl protein extract was mixed with 5 mL Bradford reagent and absorbance read at 595 nm. Extracts were spotted on $60F_{254}$ TLC plates and developed in 1-butanol-glacial acetic acid-water (4:1:1). TLC plates were sprayed with ninhydrin solution and dried in an oven at 110°C to visualise the spots.

Quantification of phenolics and flavonoids

Watermelon seed powders, 2.0 g, were quantified for total phenolics. Phenols were determined using Folin-Ciocalteu protocol as described by Torres et al. (1987). The analyses were done in triplicates using gallic acid as a standard. The amount of total phenolics was calculated in mg gallic acid equivalents/g dry seed material. For the determination of flavonoids, 500 µl of phenolic extracts was analysed using a protocol by Torres et al. (1987). Total flavonoids were determined as catechin equivalent from the calibration curve of catechin standard solutions and expressed as mg catechin equivalents/g seed material.

Total potential antioxidant activity

The antioxidant activity on watermelon seed extracts was based on their scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals according to a modified assay by Odhav et al. (2007). To determine the time course of antioxidant activity, phenolic extracts were mixed with 200 µM DPPH and allowed to stand for 10 min before absorbance was read at 517 nm. An absorbance versus time graph was plotted to determine the time course of inhibition.

Ash, crude fibre and carbohydrate analysis

Ash content was determined by heating 5.0 g seed powder in porcelain crucibles under a muffle furnace at 500°C for 2 h. The weight of the residual ash content was then calculated as percentage ash content: Ash content (%) = weight of ash / weight of original sample × 100%. Crude fibre was determined by boiling defatted seed powder in 1.3% aqueous sulphuric acid (H_2SO_4) and boiled for 30 min. The filtrate obtained was boiled in 2.5% sodium hydroxide (NaOH) for 30 min. The solution was filtered and residues transferred into a crucible and dried at 105°C to a constant weight. The crude fibre content percentage was calculated as: Crude fibre (%) = weight of dry residue - weight ash / weight of defatted seed material × 100%. The carbohydrate content was determined using the method described by Striegel and Hill (1996) using a portable refractometer REF113 Brix 0-32 ATC for the sugars.

Mineral analysis

The mineral elements, comprising of calcium, magnesium, sulphur, iron, zinc and copper were quantified according to Inuwa et al. (2011). The mineral solution obtained from ashed materials was

Table 1. Fruit and seed physical characteristics of the nine watermelons.

Landrace	Mass per 100 seeds (g)	Colour of fruit	Seed colour
01SDPW	8.5±0.141^{g}	Green with cream stripes	Black with brown speckles
02SDPW	8.35±0.778^{h}	Green	Black with brown edges
03SDPW	8.55±0.495^{f}	Green	Brown with cream stripes
05SDPW	9.25±0.495^{d}	Greenish-Grey	Light brown
06CDGM	10.95±0.071^{a}	Green	Black with brown edges
07CDGM	8.05±0.212^{i}	Green with cream stripes	Black with brown edges
08CDGM	10.55±0.919^{b}	Greenish-Grey	Light brown with black speckles
09CDGM	9.35±0.636^{c}	Greenish-Grey	Light brown with black tips
10CDGM	8.7±0.141^{e}	Green	Black with brown edges

Values are means with standard deviation of three measurements. Values followed by the different letters are significantly different at $p \leq 0.05$ confidence level.

Table 2. Phytochemical characteristics of crude oil of the watermelon seed oils.

Landrace	Oil yield (g)	Saponification value	Iodine value	Oil (%)	Seed oil colour
01SDPW	3.00±0.14^{e}	132.40±0.793^{e}	136.798±0.449^{f}	30.0±0.14^{a}	Orange
02SDPW	3.25±0.21^{c}	184.57±2.380^{c}	134.006±0.269^{a}	32.5±0.21^{b}	Golden Yellow
03SDPW	3.25±0.07^{c}	143.06±1.587^{c}	135.085±1.077^{e}	32.5±0.07^{a}	Orange
05SDPW	3.15±0.35^{d}	148.95±0.397^{d}	137.687±0.269^{d}	31.5±0.35^{a}	Orange
06CDGM	3.55±0.07^{a}	154.84±0.793^{a}	135.149±0.628^{c}	35.5±0.07^{a}	Golden Yellow
07CDGM	3.00±0.00^{e}	135.20±3.967^{e}	134.704±0.359^{f}	30.0±0.00^{b}	Orange
08CDGM	3.35±0.21^{b}	161.29±1.190^{b}	138.194±0.090^{b}	33.5±0.21^{a}	Golden Yellow
09CDGM	3.15±0.49^{d}	145.30±2.380^{d}	138.575±0.269^{a}	31.5±0.49^{a}	Pale Yellow
10CDGM	4.15±0.35^{d}	145.02±3.570^{d}	136.862±0.538^{d}	41.5±0.35^{a}	Orange

Oil yield was expressed as g/10 g sample. Saponification value was measured in mg KOH/g oil, and Iodine value in grams of I_2/100 g oil. Values with same superscript letters are not significantly different within the column and ± values are standard deviations of triplicates.

filtrated and used to determine trace elements (iron, zinc and copper) using Varian Spectra AA atomic absorption spectrometer. Sulphur was determined gravimetrically as barium sulphate ($BaSO_4$) (Mendham et al., 2000). Calcium-magnesium complex was determined by complexiometric titrimetric analysis (Mendham et al., 2000). Crystals of eriochrome black T were used as indicator with the end point being a colour change from wine red to clear blue.

Statistical analysis

Analysis of variance (ANOVA) was done using SPSS version 22 (IBM SPSS Statistics). Results reported in the tables were obtained from three replicates (n=3) and are reported as means ± standard deviation of the mean. Data was used to determine the significance level at p value<0.05.

RESULTS AND DISCUSSION

The results indicate that, Capricorn and Sekhukhune watermelons differed significantly in their morphology and chemical compositions. All fruits were globular with shallow grooves and the skins appeared light to deep green with creamy stripes in colour. The seeds were small, black in colour with crown stripes and brown with creamy white stripes, for the Capricorn and Sekhukhune districts respectively. These characteristics which are typical of the Cucurbitaceae may be due to the genotypes and environmental conditions. The difference in seed mass observed in 06CDGM (10.95 g) was ascribed to bigger seed size and thus demonstrating higher amounts of phytochemicals probably contained within the seeds (Tables 1 and 2). Mariod et al. (2009) reported a positive correlation between seed size and amounts of oils, proteins, carbohydrates and fibre found in the seeds.

Ash, fibre and carbohydrate proximate composition

As shown in Figure 1, the highest estimated percentage compositions for crude fibre, ash and carbohydrate

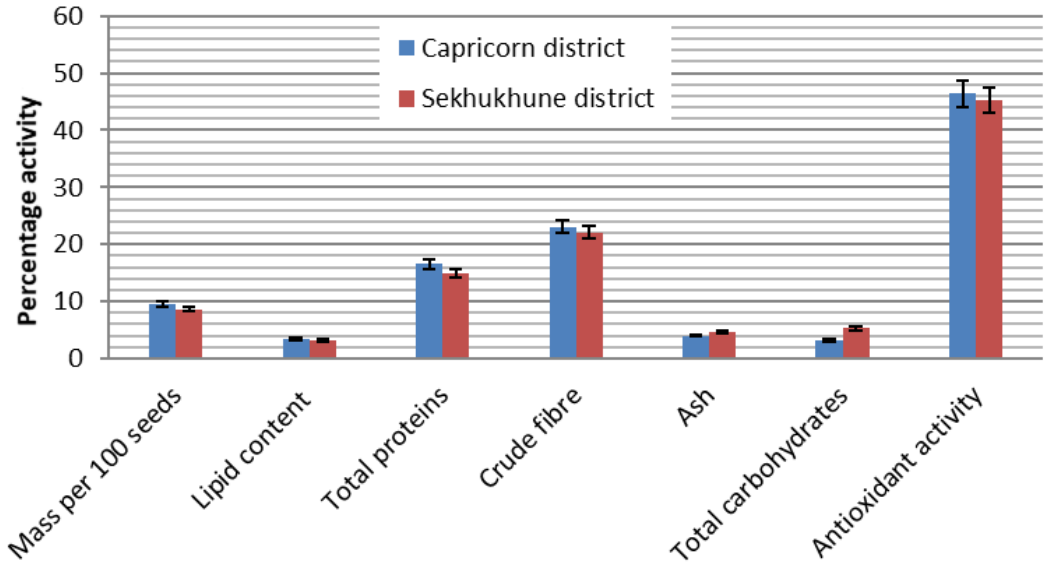


Figure 1. Quantification of the antioxidant activity and nutrient composition of watermelon landraces collected from two districts in Limpopo.

content were 23.1, 4.58 and 5.26%, respectively. Capricorn landraces exhibited high crude fibre (23.1%) than Sekhukhune varieties which had high ash (4.58%) and sugar (5.26%) content. The sugar content obtained in this study was found to be very low as compared to 22.9 and 26.6% reported for other watermelons including *Cucumis melo* (Mehra *et al*. 2015). Ash serves as inorganic content from which mineral elements are obtained. As expected, landraces from Sekhukhune were found to contain higher Fe, Zn, S, Ca and Mg. These findings are favourably comparable with observations made by Tabiri et al. (2016) even though their ash percentages were relatively low (2.0 to 3.0%). Landraces differed in the amount of copper, sulphate and iron. Furthermore, the high ash and fibre indicate the potential to provide higher amounts of minerals. Further research could lead to use of these seeds as good source of essential nutrients used as supplements in manufacturing of foods and feed products.

Quantity and physical characteristics of crude oils

Landraces collected from Capricorn district contained high amounts of oil (34.4%) than Sekhukhune varieties (31.6%) (Table 2). The oil content in the samples significantly differed ($p \leq 0.05$) among all the landraces. Variety 10CDGM was the highest with 4.15 g, followed by 06CDGM with 3.55 g. The lowest yields obtained from 01SDPW and 07CDGM both recorded 3.00 g oil. The colour of oil obtained in 10CDGM was orange (Table 2) showing impurities as reported by Raziq et al. (2012). Light yellow oil is considerable and the physicochemical analysis indicated commensurate saponification and iodine values for other similar oilseed such as pumpkin and *Cucumis* oil (Ibeto et al., 2012).

The lighter the oil, the more valuable and acceptable the oil is as edible vegetable oil. However, the variety with the highest oil mass did not exhibit the highest saponification and iodine values. The saponification value was lower as compared to other oilseeds such as soybean (191 mg KOH/g oil) and sunflower (194 mg KOH/g oil) (Baboli and Kordi, 2010). Generally, watermelons collected from Sekhukhune had higher saponification value but lower iodine value. Such indications imply that the seed oil may be low affecting antioxidants, thus reduced potential use for industrial and medicinal purposes. This variation in the seed oils may have been attributed to environmental factors as indicated by Maranz and Weisman (2003) and Di-Vincenzo et al. (2005).

Protein and amino acid composition

The amount of crude protein content obtained was between 14.1 and 20.4%. Capricorn landraces had 16.5% protein content as compared to samples from Sekhukhune district (14.9%). These amounts were very low when compared with 14.5 to 33.3% reported by Karanja et al. (2013) on other *Cucurbita* species. Furthermore, observations compared poorly with seeds of cashew nuts (22.8%), *Cucumis sativus* (26.7%), and *Cucurbita pepo* (27.5%) reported by Karanja et al. (2013).

The amounts of amino acids varied significantly and leucine was found to be the most abundant amino acid. In comparison, Usman et al. (2010) reported the prevalence of glutamic acid and aspartic acid in watermelon seeds. This implies that variation occurs according to genotype and the geographical and environmental conditions in which watermelons are grown. Threonine was the second most abundant, detected in all landraces except in 01SDPW. The prevalence of methionine was low in landraces from Sekhukhune but was high for Capricorn.

This multi-functional sulfur-containing amino acid plays an important role in the synthesis of essential proteins and stabilisation of specific interactions within proteins and protein complexes. The high abundance of this essential amino acid demonstrates the potential role of watermelon seeds in human biological activities. Therefore, observations indicate that watermelons have attractive amino acid and protein profiles, providing greater prospective for use as food supplements.

Total phenolics, flavonoids and antioxidant activity

Flavonoid content in the watermelon seeds was in the range of 0.015 to 0.347 mg/g of sample. Capricorn landraces had high flavonoid content (0.222 mg/g) than Sekhukhune varieties (0.130 mg/g). Adetutu et al. (2015) reported 0.05 mg/g of flavonoids in watermelon seeds. In contrast, henna, pomegranate and soybean seeds reportedly contain 1.10, 6.79 and 0.580 mg/g, respectively (Cherbi et al., 2016; Elfalleh et al., 2012; Josipovic et al., 2016). However, phenolics quantified were found to be significantly lower than previously reported by other researchers. About 0.57 mg gallic acid equivalents/g was obtained. This is much lower as compared to reports by Etim et al. (2013) and Sharma et al. (2013) with an average of 0.96 mg gallic acid equivalents/g in other *Cucurbita* spp. Xu and Chang (2008) reported fewer flavonoids and phenolics in soybean.

Varghese et al. (2013) reported that the different techniques employed affect the results and also limit the observations, rendering them somehow incomparable. Therefore, differences observed in our study may also be due to the extraction conditions, as well as the genotypes. Antioxidant activity expressed as %DPPH inhibition of the landraces is presented in Figure 1. Capricorn varieties had high scavenging capacity (46.5%) than Sekhukhune (45.3%). Variation in DPPH activity may be due to the high phenolic content found in Capricorn extracts. Rahman et al. (2013) stated that phenols are responsible for high scavenging activity than other phytochemicals. About 56.9% DPPH free radical scavenging activity of watermelons seeds was reported by Oseni and Okoye (2013). Tabiri et al. (2016) reported 94.46, 70.06 and 59.88% scavenging ability of watermelons cultivar Crimson sweet, Black diamond and Charleston gray, correspondingly.

Conclusions

The results suggest that watermelons from Capricorn district have better phytochemicals and antioxidant capacity than those collected from Sekhukhune district. These results further indicate that seed extracts from these watermelon landraces contain relatively high amounts of oil and secondary metabolite contents coupled with a fair antioxidant activity and thus, suitable to enhance the nutritional quality of foods and feeds.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Addo PW, Agbenorhevi JK, Adu-Poku D (2018). Antinutrient contents of watermelon seeds. MOJ Food Processing and Technology 6(2):237-239.

Adetutu A, Olorunnisola OS, Owoade OA (2015). Nutritive value and antioxidant activity of *Citrullus lanatus* fruit extract. Food and Nutrition Sciences 6:1056-1064.

Association of Official Analytical Chemists (AOAC) (2006). Official Method 982.30. Association of Official Analytical Chemists International, Washington.

Baboli ZM, Kordi AAS (2010). Characteristics and composition of watermelon seed oil and solvent extraction parameters effects. Journal of the American Oil and Chemists Society 87:667-671.

Badlfu GIO (1991). Chemical and physical analyses of oils from four species of *Cucurbitaceae*. Journal of the American Oil Chemists Society 68:428-432.

Cherbi R, Yousfi M, Saidi M, Belguidoum M (2016). Total phenols, flavonoid content and antioxidant activity of seeds extracts of *Lawsonia alba* (henna) from Algeria. Journal of Pharmaceutical Chemistry, Medical Chemistry and Chemical Sciences 8:216-221.

Chiang CJ, Kadouh H, Zhou K (2013). Phenolic compounds and antioxidant properties of gooseberry as affected by *in vitro* digestion. Food Science and Technology 51:417-422.

Choudhury BR, Haldhar SM, Maheshwari SK, Bhargava R, Sharma SK (2015). Phytochemicals and antioxidants in watermelon (Citrullus lanatus) genotypes under hot arid region. Indian Journal of Agricultural Sciences 85(3):414-417.

Devasagayam TPA, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD (2004). Free radicals and antioxidants in human health: Current status and future prospects. Journal of the Association of Physicians of India 52:794-804.

Di-Vincenzo D, Maranz S, Serraiocco A, Vito R, Weisman Z, Biachi G (2005). Regional variation in shea butter lipid and triterpene composition in four African countries. Journal of Agriculture and Food Chemistry 53:7473-7479.

Edwards AJ, Vinyard BT, Wiley ER, Brown ED, Collins JK, Perkins-Veazie P, Baker RA, Clevidence BA (2003). Consumption of watermelon juice increases plasma concentrations of lycopene and beta carotene in humans. Journal of Nutrition 133(4):1043-1050.

Elfalleh W, Hannachi H, Tlili N, Yahia Y, Nasri N, Ferchichi A (2012). Total phenolic contents and antioxidant activities of pomegranate

peel, seed, leaf and flower. Journal of Medicinal Plants Research 6:4724-4730.
Etim OE, Ekanem SE, Sam SM (2013). *In vitro* antioxidant activity and nitric oxide scavenging activity of *Citrullus lanatus* seeds. Journal of Natural Science Research 3:126-132.
George KO, Kinyanjui T, Wanyoko J, Moseti OK, Wachira F (2013). Extraction and analysis of tea (*Camellia sinensis*) seed oil from different clones in Kenya. Africa Journal of Biotechnology 12:841-846.
International Association of Fish Meal Manufacturers (IAFMM) (1981). Recommended method of analysis for determination of iodine value of fish oils (Wijs Method). International Association of Fish Meal Manufacturers. London 4:1-4.
Ibeto CN, Okoye COB, Ofoefule AU (2012). Comparative study of the physicochemical characterisation of some oils as potential feedstock for biodiesel production. International Scholarly Research Network-Renewable Energy 2012:1-5.
Josipovic A, Sudar R, Sudaric A, Jurkovic V, Kocar MM, Kulundzic AM (2016). Total phenolic and total flavonoid content variability of soybean genotypes in eastern Croatia. Croatian Journal of Food Science and Technology 8:60-65.
Inuwa HM, Ania VO, Gabi B, Aimola I, Thompson V (2011). Determination of differences in nutrient composition of *Citrullus vulgaris* (watermelon) fruits after plucking. British Journal of Dairy Science 104:127-139.
Karanja JK, Mugendi BJ, Khamis FM, Muchugi AN (2013). Nutritional composition of the pumpkin (*Cucurbita* spp.) seed cultivated from selected regions in Kenya. Journal of Horticulture Letters 3:17-22.
Maranz S, Weisman Z (2003). Evidence for indigenous selection and distribution of the shea tree, *Vitellaria paradoxa*, and its potential significance to prevailing parkland savanna tree patterns in sub-Saharan Africa north of the equator. Journal of Biogeography 30:1505-1516.
Mariod AA, Ahmed YM, Matthaus B, Khaleel G, Siddig A, Gabra AM, Abdewahab SI (2009). A comparative study of the properties of six Sudanese cucurbit seeds and seed oils. Journal of the American Oil Chemists Society 86:1181-1188.
Mehra M, Pasricha V, Gupta RK (2015). Estimation of nutritional, phytochemical and antioxidant activity of seeds of musk melon (*Cucumis melo*) and water melon (*Citrullus lanatus*) and nutritional analysis of their respective oils. Journal of Pharmacognosy and Phytochemistry 3:98-102.
Mendham J, Denny RC, Barnes JD, Thomas MJK (2000). Vogel's Textbook of Quantitative Chemical Analysis. 6th Edition. Prentice Hall, London.
Najafi S, Sanadgol N, Nejad BS, Beiragi MA, Sanadgol E (2010). Phytochemical screening and antibacterial activity of *Citrullus colocynthis* (Linn.) Schrad against *Staphylococcus aureus*. Journal of Medical Plants Research 4(22):2321-2325.
Odhav B, Beckrum S, Akula A, Baijnath H (2007). Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal South Africa. Journal of Food Composition and Analysis 20:430-435.
Oseni OA, Okoye VI (2013). Studies of phytochemical and antioxidant properties of the fruits of watermelons (*Citrullus lanatus*). Journal of Pharmaceutical and Biomedical Sciences 27:508-514.
Oyeleke GO, Olagunju EO, Ojo A (2012). Functional and physicochemical properties of watermelon (*Citrullus lanatus*) seed and seed-oil. IOSR Journal of Applied Chemistry 2:29-31.
Rahman H, Manjulak K, Anoosha T, Nagaveni K, Chinna EM, Dipankar B (2013). *In-vitro* antioxidant activity of *Citrullus lanatus* seed extracts. Asian Journal of Pharmaceutical and Clinical Research 6:152-157.
Raziq S, Anwar F, Mahmood Z, Shahid SA, Nadeem R (2012). Characterization of seed oils from different varieties of watermelon [*Citrullus lanatus* (Thunb.)] from Pakistan. Grasas y Aceites 63:365-372.
Sharma A, Sharma AK, Chand T, Khardiya M, Yadav KC (2013). Preliminary phytochemical evaluation of seed extracts of *Cucurbita maxima* Duchesne. Journal of Pharmacognosy and Phytochemistry 2:62-65.
Striegel MF, Hill J (1996). Thin-Layer Chromatography for Binding Media Analysis: Scientific Tools for Conservation. The J. Paul Getty Trust, USA http://d2aohiyo3d3idm.cloudfront.net/publications/virtuallibrary/0892363908.pdf
Tabiri B, Agbenorhevi JK, Wireko-Manu FD, Ompouma EI (2016). Watermelon seeds as food: Nutrient composition, phytochemicals and antioxidant activity. International Journal of Nutrition and Food Sciences 5:139-144.
Torres AM, Mau-lastovicka T, Rezaainyan R (1987). Total phenolics and high-performance liquid chromatography of phenolics in avocado. Journal of Agriculture Food Chemistry 35:921-925.
Usman A, Haruna M, Itodo V, Oforghor OA (2010). Protein and amino acid composition of water melon (*Citrullus lanatus*) seeds. International Journal of Tropical Agriculture and Food Systems 4:343-356.
Varghese S, Narmadha R, Gomathi D, Klaiselvi M, Devaki K (2013). Phytochemical screening and HPTLC finger printing analysis of Citrullus lanatus (Thunb.) seed. Journal of Acute Disease 2:122-126.
Xu B, Chang SK (2008). Antioxidant capacity of seed coat, dehulled bean, and whole black soybeans in relation to their distributions of total phenolics, phenolic acids, anthocyanins, and isoflavones. Journal of Agriculture and Food Chemistry 56:8365-8373.

Related Journals: