STRUCTURAL, CHEMICAL AND PHYSIOLOGICAL INVESTIGATIONS OF BILIRUBIN FOUND IN SEED ARILS OF *Strelitzia nicolai*

By

DEPIKA DWARKA

Submitted in fulfilment of the academic
Requirements for the degree of
Doctor of Philosophy in the
School of Life Sciences,
University of KwaZulu-Natal
Westville campus

January 2017

As the candidate's supervisors we have approved this thesis for submission.

Supervisor:
Signed: _____ Name: Prof H Baijnath Date: _____

Co-supervisor:
Signed: _____ Name: Dr Veneesha Thaver Date: _____

Co-supervisor:
Signed: _____ Name: Dr Mickey Naidu Date: _____

DEDICATION

To my late husband, Mr Leon Subramanien and my late grandmother, Mrs Suminthra Dwarka, both who passed on during the course of this study.

"*Wear your tragedies as Armor, not shackles*"

ABSTRACT

In 2009, a team of researchers from the Florida International University discovered bilirubin which is an animal compound in the seed arils of *Strelitzia nicolai*, commonly known as the White Bird of Paradise plant. Bilirubin is an endogenous yellowish compound that is formed following the standard catabolic pathway by the breakdown of haem. This catabolism is an essential practice in the human body as it allows for the elimination of waste products. Bilirubin is formed in humans, some vertebrates and invertebrates. Previously thought to be a toxic compound if accumulated, recent advancements in medical sciences show bilirubin as an effective antioxidant. This study describes novel protocols to reveal new insights into the presence of the only animal pigment found in *S. nicolai* arils, the potential advantages of bilirubin found in a plant and its therapeutic value indications. *In vitro* and *in vivo* experiments using the aril extract was conducted in order to assess the antioxidant, anti-cancer and toxicity dosage of *S. nicolai* aril extract. *In vitro* studies showed that *S. nicolai* aril extract caused apoptosis in 52% of Hela cancer cell lines. These results indicate that *S. nicolai* arils extract possess conceivable chemo preventive properties. *In vivo* results in a rat model showed no acute toxicity nonetheless, these results were inconclusive as a lengthier chronic study needs to be conducted in order to completely rule out toxicity. An ultrastructure study of the seed arils was embarked in order to integrate supplementary knowledge. The developing seeds were grouped in five stages according to seed maturity and aril colour. The arils were analysed using light and electron microscopy. The Hall's staining which is generally used on human tissue was modified to detect bilirubin on the surface of the aril tissue. Light microscopic results showed that as the aril matures it undergoes an atypical colour change from opaque to yellow and finally a deep orange. This colour change might be attributed to the accumulation of bilirubin in the aril tissue. This hypothesis was further justified by cryo-SEM and TEM which shows the trend of an accretion of a pigment as the aril matures. In addition, the inclination of aril maturity in *S. nicolai* follows an analogous prototypical pathway to that of bilirubin catabolism in humans. This study consequently highlights the potential use of *S. nicolai* aril extract containing bilirubin and also attempts to relate aril structure with impending function.

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, from August 2013 to December 2016, under the supervision of Prof Himansu Baijnath and co-supervision of Dr Mickey (GC) Naidu and Dr Veneesha Thaver.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION 1 - PLAGIARISM

I, Ms Depika Dwarka declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: ……

DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1

Depika Dwarka[1], Veneesha Thaver[2], Mickey Naidu[3] and Himansu Baijnath[4]. 2017. New insights into the presence of bilirubin in a plant species *Strelitzia* (Strelitziaceae). *African Journal of Traditional, Complementary and Alternative Medicine* 14 (2): 253-262.

Contributions: Depika carried collected data and wrote manuscript. Dr Thaver, Dr Naidu and Prof Baijnath edited the manuscript.

Publication 2

Depika Dwarka[1], Veenesha Thaver[2], Mickey Naidu[3], Neil A. Koorbanally[4] and Himansu Baijnath[5]. 2017. In *vitro* chemo-preventative activity of *Strelitzia nicolai* aril extract containing bilirubin. *African Journal of Traditional, Complementary and alternative medicine* 14 (3): 000-000

Contributions: Depika carried out experimental work, recorded data and wrote manuscript. Dr Thaver, Dr Naidu, and Prof Baijnath were the supervisors and provided comments. Dr Koorbanally allowed the use of his facilities and provided comments.

Publication 3

Depika Dwarka[1*], Sanil Singh[2], Veneesha Thaver[3], Mickey Naidu[3] and Himansu Baijnath[4*]. Effect of *Strelitzia nicolai* seed arils containing bilirubin on sprague dawley rats. Submitted to *Laboratory Animals*

Contributions: Depika carried out experimental work, recorded data and wrote manuscript. Dr Thaver and Prof Baijnath were the supervisors and provided comments. Dr Singh allowed the use of his facilities. Dr Singh and Dr Naidu assisted with the experimental design.

Publication 4

Depika Dwarka[1*], Nelisha Murugan[2], Veneesha Thaver[3], Mickey Naidu[3] and Himansu Baijnath[4*]. Preparation techniques of *Strelitzia nicolai* aril tissue for scanning electron microscopy. Submitted to *Microscopy, Research and Techniques*

Contributions: Depika carried out experimental work, recorded data and wrote manuscript. Dr Thaver, Dr Naidu and Prof Baijnath were the supervisors and provided comments. Ms Murugan allowed for the use of the MMU, assisted with experimental design and editing of the manuscript.

Publication 5

Depika Dwarka[1*], Nelisha Murugan[2], Veneesha Thaver[3], Mickey Naidu[3] and Himansu Baijnath[4*]. A comparative microstructural study of the animal pigment, bilirubin, in seed arils of *Strelitzia nicolai.* Submitted to *Experimental Botany*

Contributions: Depika carried out experimental work, recorded data and wrote manuscript. Dr Thaver, Dr Naidu and Prof Baijnath were the supervisors and provided comments. Ms Murugan allowed for the use of the MMU, assisted with experimental design and editing of the manuscript.

Signed:_____

ACKNOWLEDGEMENTS

It is my pleasure to thank the many people who made this thesis possible

- Prof Himansu Baijnath has been an ideal supervisor. His sage advice, insightful criticisms and patience, aided this study in innumerable ways
- Dr Mickey Naidu whose steadfast support of this project was greatly needed and deeply appreciated
- Dr Veneesha Thaver for her constructive critics and guidance
- The Microscopy and Microanalysis Unit, The Department of Physiology and The Biomedical Resource Unit, UKZN, has made available their support in a number of ways
- I am deeply indebted to many of my colleagues who supported me during this study especially Ms Nelisha Murugan, Mr Vishal Bharuth, Mr Subashen Naidu, Dr Kogie Moodley, Dr Sanil Singh, Mr Preenan Pillay, Ms Sohana Singh and Ms Karishma Singh
- My brother, Mr Shaun Dwarka, has been always, my pillar and my guiding light
- My babies, Kayur and Kee-aryan, have been a constant source of emotional support and my motivation to make the impossible; possible
- Lastly, and most importantly, my parents. They raised me, supported me, educated me and loved me. Without them this thesis would not have been possible

TABLE OF CONTENTS

Chapter 1

Introduction

In the late 18th century, bilirubin was discovered as a component of bile by Jean Baptiste Thimotee Baumes (Baumes, 1806). Since the primeval era, scientists have considered bile to be one of the four vital components of the body that manage overall health (McCandless, 2011). Additional curiosity in the configuration of bilirubin was stimulated by the illness identified as 'jaundice'. Augustine Nicholas Gilbert was one of the key pioneers who established that increased plasma bilirubin was hereditary (Horsfall et al., 2013). Whilst the significance of bilirubin as a crucial component of bile was recognized, there was nothing known about bilirubin's formation in the human body. One of the primary innovations that contributed to knowledge on bilirubin's creation was by Hiroshi Nakajiima who characterized the enzyme α-menthenyl oxygenase that transformed haem to a precursor of bilirubin (Stec, 2011).

Bilirubin is not only established in humans but can also be found in an extensive selection of animals (Table 1). In mammals, levels of biliverdin are metabolized to bilirubin whereas; in other animals e.g. fish, only a portion of the biliverdin is metabolized to bilirubin (Fang and Bada, 1990). In 2009, bilirubin was unexpectedly discovered in several plants. This breakthrough became known as the only human compound to be found in a plant. Pirone et al., (2010) found a large quantity of bilirubin in the arils of *Strelitzia nicolai* Regel and Körn.

Extremely precise enzyme particle mechanisms are needed in order to produce bilirubin. As other plants do not contain bilirubin, Rouserev and Martin (2009) explain that convergent evolution where the same property is considered to have evolved independently in different organisms or the lateral transfer of genes could be responsible for the production of this human compound in a plant.

Table 1: Animals that produce bilirubin

Invertebrates	Vertebrates
Protozoa – Absents	Fish – present in some fish e.g. salmon
Echinoderms - Absent	Amphibians – present in some amphibians
Annelids – present in some	Reptiles - Absent
Molluscs – present in some	Birds - Absent
Arthropods - Absent	Mammals - present
Crustaceans - Absent	Marsupials - present
Arachnids - Absent	Primates - present
Insects – Absent, contains Biliverdin	Rodents - present
	Cetaceans – present

1.1 Bilirubin: Formation, metabolism, and excretion

The production of bilirubin and its removal from the body as a desecrate product of haem catabolism entail a sequence of metabolic modification and transfer procedures (as illustrated in Figure 1). As the red blood cells collapse, the haemoglobin is broken down into globin and haem. The haem firstly breaks away from each other into biliverdin, a green pigment which is rapidly condensed to bilirubin, an orange-yellow pigment (Ewing and Maines, 1997). These processes all takes place in the liver, spleen and bone marrow cells. The bilirubin is then chelated into bile. Bilirubin is excreted from the body by a conversion in the bladder and intestine to pigments i.e. stercobilin and urobilinogen and is finally expelled as standard constituents of the urine and faeces (Ophardt, 2003).

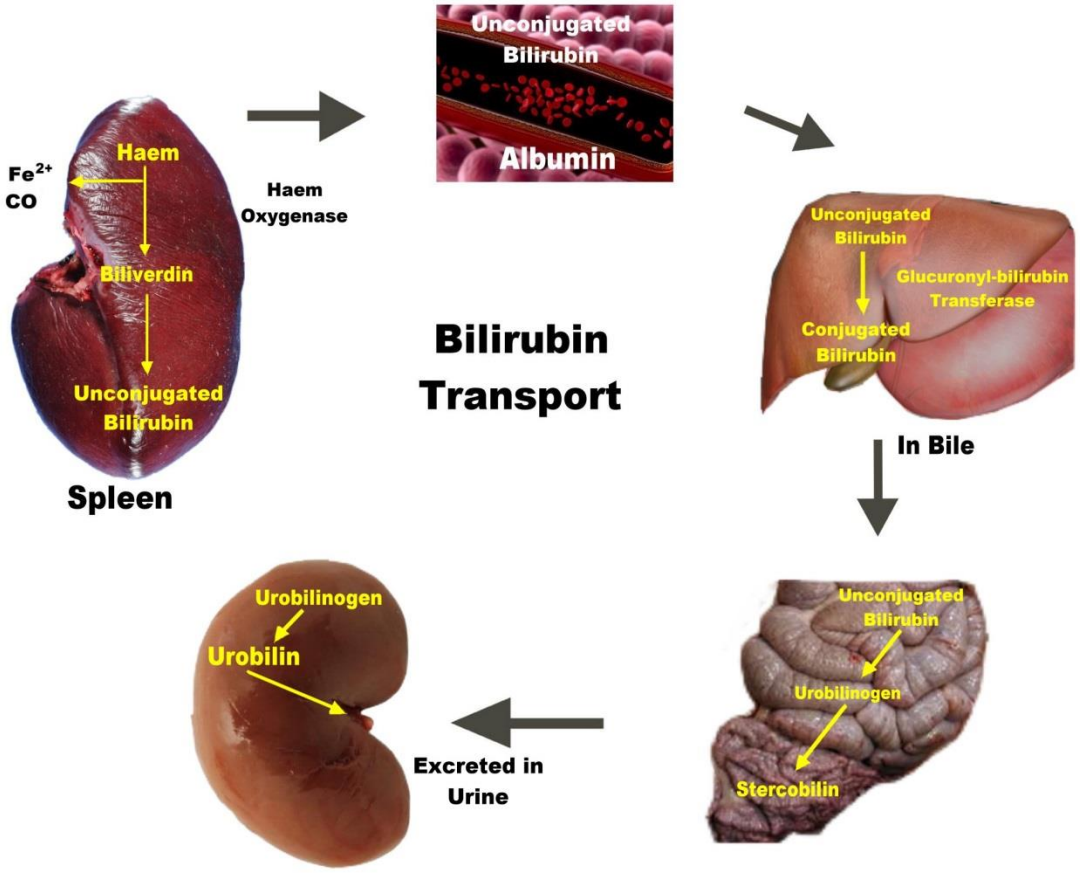


Figure 1: Transportation and excretion of bilirubin in humans (adapted from Ophardt, 2003).

1.2 Effect of disturbances in bilirubin metabolism

Bilirubin, if not metabolized effectively can lead to accumulation. The disorder infirmities of bilirubin metabolism (Table 2) can be separated into four key categories: explicitly those caused by (a) excessive pigment production, (b) decreased hepatic uptake of bilirubin, (c) dysfunctional hepatic conjugation and (d) reduced excretion of the conjugated pigment from the liver into the gall bladder, intestines and kidney. The initial three disorders are linked to principally unconjugated hyperbilirubinemia. The fourth set, poor excretion, is related to primarily conjugated hyperbilirubinemia and bilirubinuria (Isselbacher et al., 1987).

Table 2: Laboratory features in icteric states (LaMont and Isselbacher, 1985)

Bilirubin disorder	Unconjugated	Conjugated	Urine bilirubin
I Overproduction			
a) Haemolysis	↑	N	0
b) Ineffective erythropoiesis	↑	N	0
II Defective hepatic uptake			
a) Some drugs			
b) Gilbert's syndrome	↑	N	0
III Defective conjugation			
a) Neonatal jaundice	↑	Low	0
b) Gilbert's syndrome	↑	Low	0
c) Crigler-Najjar syndromes	↑	Low	0
IV Defective excretion			
a) Intrahepatic obstruction (Dublin Johnson, Rotor)	↑	↑	+
b) Extrahepatic obstruction (tumors, stones)	↑	↑	+
V Hepatocellular disease			
a) Hepatitis	↑	↑	+
b) Cirrhosis	↑	↑	

** ↑ denotes increase, N denotes No, + denotes present and 0 denotes absent.*

1.3 Biological Properties of bilirubin

Bilirubin is integrated to the superfamily of tetrapyrrolic amalgam. This group is made up of preserved molecules in nature. For decades, bilirubin was thought to be a latently lethal metabolite of the haem catabolic pathway. Findings from recent studies suggest that bilirubin at slightly elevated levels could be beneficial. Vitel (2012) confirmed that bilirubin could be favourable for the scavenging of overproduced reactive oxygen species, anti-inflammatory actions and to direct effects upon cell signalling.

Bilirubin has recently been acknowledged as a chemical with powerful antioxidant properties. Stocker et al., (1987) confirmed the "pharmacotherapeutic" role of bilirubin as a physiological antioxidant. They confirmed that micromolar concentrations *in vitro* efficiently scavenge peroxyl radicals.

In recent years, inverse relationships between serum bilirubin concentration and irregular glucose tolerance analysis were reported (Ko et al., 1996). Furthermore, bilirubin was also reported to offer protection against metabolic syndromes and to be negatively related to overweight and obesity (Vitek, 2012). Bilirubin also plays an important role in immunomodulatory and anti-inflammatory activity (Paine et al., 2010). As from the metabolic outlook, there are a number of vital enzymatic steps that play an imperative task in bilirubin homeostasis with successive impacts on the threat of metabolic diseases, including cardiovascular diseases, diabetes, metabolic syndrome, arterial hypertension and obesity (Vitek, 2012).

The use of animal bile in China for the treatment of a broad number of disorders in humans dates back to 2500 years ago (Chen, 1987). Although forty-four animal bile (including human bile) have been used as highly accepted drugs in ancient Chinese traditional medicine only bile from pig, ox and bear are extensively employed in China today (Wang and Carey, 2014). Scientists believed that specific bile salts as well as the bile pigment bilirubin are useful in improving liver function and dissolving gallstones. They also serve as potent antibacterial, virucide, anti-inflammatory, anti-pyretic, antioxidant, sedative, anti-convulsive, anti-allergic, anti-congestive, anti-diabetic and anti-spasmodic agents (Table 3).

Table 3: List of diseases treated with animal bile in traditional Chinese medicine (Wang and Carey, 2014)

Disease	Animal bile or gallstone
Digestive system	
Jaundice	bear, ox
Biliary colic	bear, python, yak
Epigastric pain	fox, gaur, kite
Gastric regurgitation	bear, dog, pig
Infantile malnutrition	bear, dog, pig, python, tiger, wild boar
Haemorrhoids	bear, duck, goose, hedgehog, ox, python, turtle
Anal fistula	bear, turtle
Diarrhoea	bear, dog, pig
Constipation	pig
Intestinal parasites	bear, ox, dog, pallas pit viper, crucian carp, horseshoe crab
Alcoholic cirrhosis	fox
Skin	
Infectious skin diseases	bear, chicken, common carp, dog, elephant, goat and sheep,
Traumatic injury	deer, dog, human, mouse
Darkish complexion	antelope, goat and sheep
Tinea versicolor	antelope, goat and sheep
Chloasma, freckle, ergsipelis	antelope, goat, and sheep
Leprosy	black snake, horseshoe crab
Eyes	
Infectious eye diseases	black carp, chicken, common carp, crow, dog, duck, goat,
Improving visual acuity	sheep,
Optic atrophy	bat, bear, common carp, crow, mouse, ox, pig, python, skink,
Night blindness	tortoise
Glaucoma and cataract	common carp, elephant, goat and sheep, mouse, python, raven
Eye injury	bat, common carp, mouse, pig, raven
	black carp, elephant
	chicken, goat, and sheep
Ears	
Suppurative otitis media	dog, mouse
Deafness	crucian carp
Nose	
Rhinitis	dog
Nasal sinusitis	bear
Rhinorrhoea	bear
Nasal polyp	dog
Mouth	
Tonsillitis	black carp, ox gallstones, shark
Gingivitis	python
Gingival atrophy	python
Dental caries	python
Halitosis	elephant
Respiratory system	
Bronchitis	ox gallstones, toad
Pneumonia	ox gallstones
Cough	ox gallstones, pig
Cardiovascular system	
Apoplexy	ox gallstones
Angina pectoris	bear
Neuropsychiatric disorders	
Epilepsy	fox, ox gallstones, wild boar
Coma	ox gallstones, fox
Endocrine system	
Diabetes mellitus	crucian carp, pig
Urinary system	
Diuresis	chicken
Cystitis	chicken
Haematuria	bear, chicken

1.4 Bilirubin found in plants

During 2010, a research panel directed by Cary Pirone from the Department of Biological Sciences at Florida International University discovered bilirubin in the well-known White Bird of Paradise plant. Pirone et al., (2010) identified the orange tincture from the arils of *S. nicolai* and used HPLC-ESMS, UV-visible, ^{1}H NMR and ^{13}C NMR to classify its chemical configuration. The results confirmed that this pigment is categorically bilirubin found in the arils of *S. nicolai*. Bilirubin was also found in low concentrations in the sepal tissue of this plant. Pirone et al., (2010) went on to further examine fruits and flowers of twelve other angiosperm species. Bilirubin was found in ten species from the Orders Zingiberales, Arecales and Myrtales.

1.5 *Strelitziaceae:* Bird of Paradise family

This angiosperm family consists of blossoming plants in the order Zingiberales (bananas, gingers and relatives). This cluster consists of three genera i.e. *Ravenala* Sonn, *Phenakospermum* A. Rich. and *Strelitzia* Aiton with seven species. *Strelitziaceae* are generally of intermediate magnitude to enormous herbs or trees (Watson and Dallwitz, 1992). Their seeds are evidently hairy by means of the aril and they are housed in a capsule. These genera are habitually found in sub-tropical to tropical regions.

Figure 2: *Strelitzia nicolai* (A) showing the whole plant with banana like leaf blades, (B) compound flower of *S. nicolai*, (C) shows boat shaped sheath containing seed capsules, (D) shows fully developed seed with bright orange aril attached, (E) shows capsules that were removed from the sheath and (F) shows matured arils detached from the seeds.

Strelitzia nicolai is commonly known as the giant white bird of paradise or wild banana tree. This tree is an evergreen that grows up to 12m. The compound flower of this wild banana contains white sepals with blue petals that are contained in blue, boat-shaped sheaths. The mature seeds are black with an orange woolly aril attached (Grant and Thomas, 1998). The ontogenetic, evolution and development of this species have been studied using electron microscopy for the past few decades (Simpson et al., 1975).

1.6 Rationale of study

Humans generate about 4 mg of bilirubin per kilogram of weight daily. This process begins with the breakdown of haem. Bilirubin, only recently has been recognized for its protective physiological role under normal concentrations, and a potent antioxidant at slightly elevated intensities (Chowdhury et al., 2001). The argument of haem being broken down into hydrophilic biliverdin and then converted into hydrophobic bilirubin opens up many questions about the existence of bilirubin. The eradication of haem could be an uncomplicated process and stops at the production of biliverdin that can be effortlessly excreted into the bile without the use of additional strength and enzymes like amphibian, reptiles and birds (McDonagh, 1979).

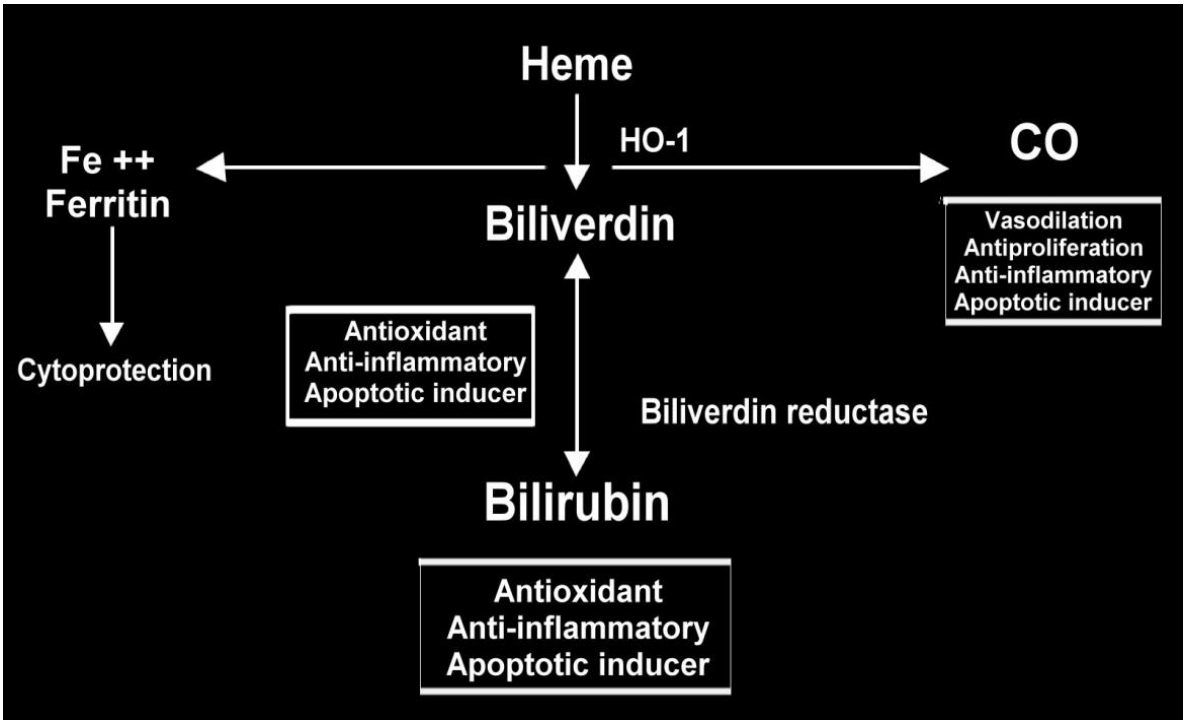


Figure 3: Potential benefits of haem metabolism (adapted from Regino et al., 2009).

The protective property of bilirubin is now acknowledged to be the underlying principle behind this energy consuming and redundant step in human metabolism. This, together with the discovery of bilirubin in a plant was the basis of the first rationale for this study.

In excess of 50% of the human brain is apprehensive with the reception and processing of optical stimuli (Gunning and Steer, 1975). With such a prominence placed on sight, humans tend to believe in something if they see it. The inadequate information about *S. nicolai* aril structure led the research to the second rationale for this study. The intent was to seek insight into the way the aril cells function, by interpreting images of their structure.

The extraction of the seed arils containing bilirubin and testing *in vitro* and *in vivo* have not been attempted previously. In this regard, the study is novel. The ultrastructure and the localization of the bilirubin found in the arils have also not been reported prior to this investigation. This study aspired to give a greater insight into the only human compound found in a plant.

1.7 Aims and Objectives of this study

Aim 1: To investigate if the bilirubin found in *S. nicolai* has a protective effect in humans and animals. To achieve this aim, the following objectives were identified:

- To extract the bilirubin found in the arils.
- To identify the bilirubin from the arils using TLC and NMR.
- To determine the antioxidant and anti-cancer potential of the aril extract compared with the bilirubin standard *in vitro*.
- To determine the toxicity of *S .nicolai* aril extract *in vivo*.

Aim 2: To examine the ultrastructure of *S. nicolai* arils. To achieve this aim, the following objectives were identified:

- To classify the arils at different stages of development.
- To possibly identify bilirubin in the arils.
- To compare the developmental phases of aril maturation and bilirubin production.
- To link the ultrastructure of the arils to function.

1.8 Outline of research presented in this thesis

In order to achieve the aims and objectives of this study, different analytical chemistry, biochemical, cell culture and microscopical techniques were used. Following the findings of the analysis, this thesis is outlined as follows: Chapter 1 gives a brief introduction to this study. Chapter 2 gives a comprehensive background into the presence of bilirubin in a human body and bilirubin found in the plant species *Strelitzia.* Chapter 3 confirms the presence of bilirubin in *S. nicolai* and thereafter evaluates the anti-cancer activity and antioxidant potential of *S. nicolai* aril extract containing bilirubin on Hela cell lines. Chapter 4 evaluates the safety of the extract *in vivo*. Chapter 5 compares the different Scanning electron microscopy preparation procedures on the quality of preservation of the aril tissue. Chapter 6 combines light and electron microscopy (TEM and cryo-SEM) in order to determine the ultrastructure of the arils at different stages of development as well as to determine the possible location of bilirubin.

The design implemented for this thesis includes chapters in the form of articles where each article has been presented in accordance to the style required by the particular journal to which the manuscript has been submitted. Only the introduction and summary of study are formatted according to the guidelines.

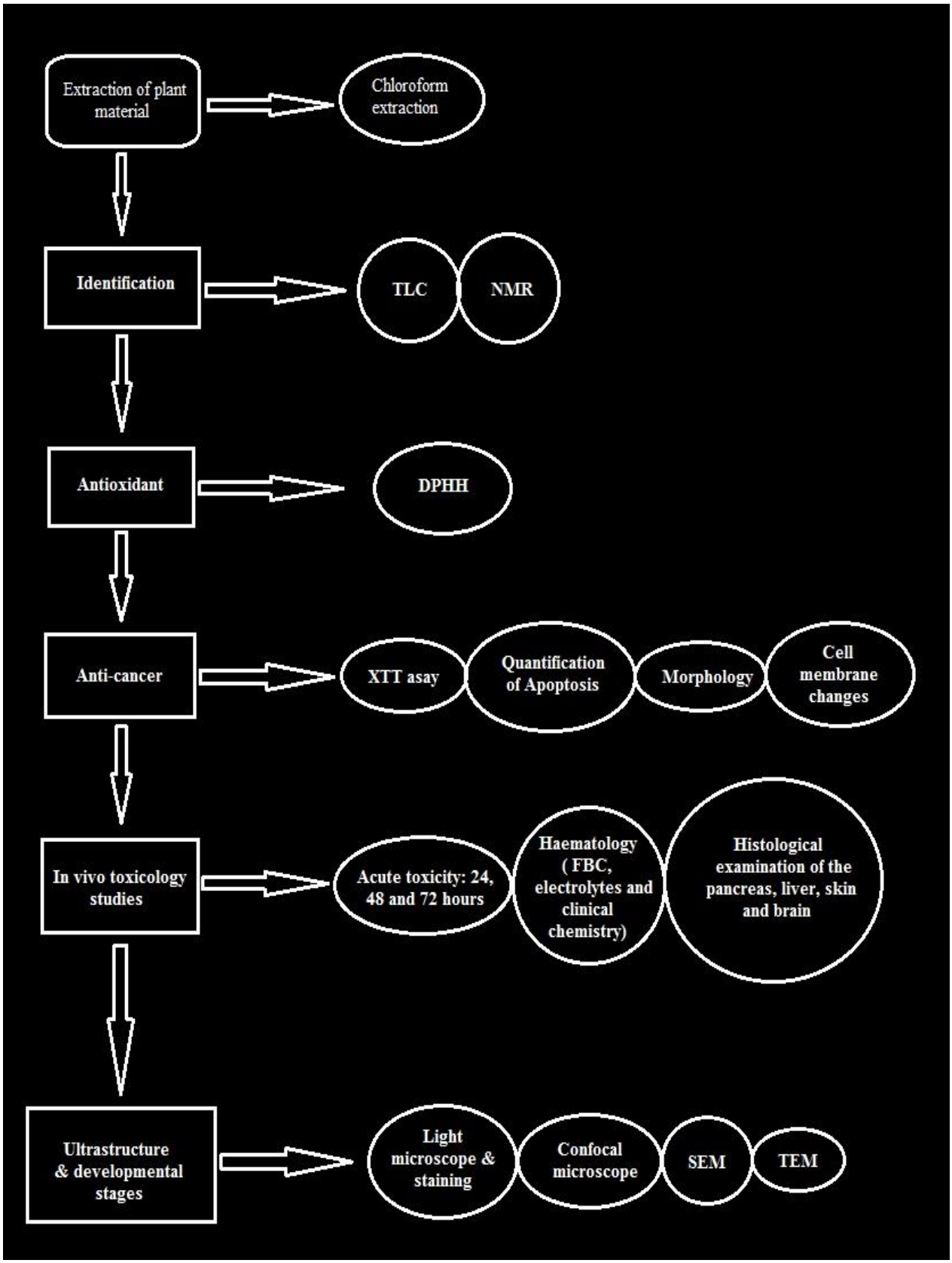


Figure 4: Outline of the methodology for this study

References

1. Baumes, T.B.T. 1806. Traite de l'amaigrisse-ment des enfans. Paris.

2. Chen, Z.Q. 1987. A supplement to materia medica. Compendium of Materia Medica. *Ben Cao Shi Yi.*

1. Chowdhury, R.J., Wolkoff, A.W., Chowdhury, N. and Arias, I. 2001. Hereditary jaundice and disorders of bilirubin metabolism: The metabolic and molecular bases of inherited disease. McGraw-Hill, London.

2. Ewing, J.F. and Maines, M.D. 1997. Histochemical localization of heme oxygenase-2 protein and mRNA expression in rat brain. *Brain Res Protoc* 1:165–174.

3. Grant, R. and Thomas, V. 1998. Sappi tree spotting KwaZulu-Natal. Jacana Publishers. Johannesburg.

4. Gunning, B.E.S and Steer, M.W. 1975. Ultrastructure and the biology of plant cells. Edward Arnold, London.

5. Horsfall, L.J, Nazareth, I., Pereira, S.P. and Petersen, I.J. 2013. Gilbert's syndrome and the risk of death: a population-based cohort study. *Gastroenterol Hepatol.* 28:10:1643-7.

6. Isselbacher, K.J., Braunwald, E., Petersdorf, R.G., Wilson, J.D., Martin, B.J. and Fauci, S. 1987. Harrison's Principles of internal medicine. McGraw Hill Inc, London.

7. Ko, G.T., Chan, J.C., Woo, J., Lau, E., Yeung, V.T., Chow, C.C., Li, J.K., So, W.Y. and Cockram, C.S. (1996). Serum bilirubin and cardiovascular risk factors in a Chinese population. *J. Cardiovasc.* 459–463.

8. LaMont, J.T. and Isselbacher, K.J. 1985. Postoperative jaundice, in liver and biliary diseases. Saunders, Philadelphia.

9. McCandless, D.W. 2011. Kernicterus. *Contemporary Clinical Neuroscience*, 11: 187-199.

10. McDonagh, A.F. 1979. Bile pigments: bilatrienes and 5, 15 biladiens.: The porphyrins. Academic Press, New York.

11. Ophardt, C. 2003. Organic and biochemistry. Virtual chembook Elmhurst college. http://chemistry.elmhurst.edu/vchembook/634bilirubin.html.

12. Paine, A., Vesper, B.E., Blasczyk, R. and Immenschuh, S. 2010. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochemical Pharmacology* 80: 12: 1895-1903.

13. Pirone, C., Quirke, M.J., Priestap, H.A. and Lee, D.W. 2009. The Animal Pigment Bilirubin Discovered in Plants. *J Am Chem Soc.* 131:8: 2830.

14. Regino, W.O., Velasco, H and Sandoval H. 2009. The protective role of bilirubin in human beings. *Rev Col Gastroenterol* 24: 3.

15. Rouserev, W.H.D. and Martin, F. 1992. Lecretius: On the nature of things. Cambridge, MA.

16. Simpson, D.J., Baqar, N.R and Lee, T.H. 1975. Ultrastructure and Carotenoid Composition of Chromoplasts of the Sepals of *Strelitzia reginaee* Aiton during Floral Development. *Annals of Botany* 39: 160. 175-183.

17. Stec, D.E. 2011. Heme Oxygenase and the kidney. Morgan & Claypool Life Sciences. Mississippi.

18. Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N. and Ames, B.N. 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* 235: 4792:1043-1046.

19. Timothy, G. 2009. Darwinism: The Science and the Faith. End time issues: A biblical perspective. http://www.endtimeissues.com.

20. Vitek, L. 2012. The role of bilirubin in diabetes, metabolic syndrome, and cardiovascular diseases. *Frontiers in pharmacology*. 3: 55: 1-7.

21. Wang, D.Q.H. and Carey, M.C. 2014. Therapeutic uses of animal biles in traditional Chinese medicine: An ethnopharmacological, biophysical chemical and medicinal review. *World J Gastroenterol* 20: 29: 9952-9975.

22. Watson, L., and Dallwitz, M.J. 1992. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. http://delta-intkey.com/angio/. Sited in October 2016.

23. Zhang, W., Zhang, R.X. and Li, J. 2015. Inheritance on and innovation of traditional Chinese medicine (TCM) flavour theory and TCM flavour standardization principle flavour theory in Compendium of Materia Medica. *Zhongguo Zhong Yao Za Zhi* 40:24:4928-31.

Chapter 2

Journal article for publication 1:

New insights into the presence of bilirubin in a plant species *Strelitzia nicolai* (*Strelitziaceae*).

Depika Dwarka[1], Veneesha Thaver[2], Mickey Naidu[3] and Himansu Baijnath[4*]

[1,4]School of Biological Sciences, University of KwaZulu-Natal, Westville Campus, Durban, South Africa. [2]School of Laboratory Medicine and Medical sciences, Discipline of Physiology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa. [3]School of Health Sciences, Discipline of Pharmacology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa.

***Email:** depika.dwarka@gmail.com, baijnathh@ukzn.ac.za

Abstract

Background: The fortuitous discovery of an animal pigment bilirubin found in the plant *Strelitzia nicolai* has opened an enormous number of questions regarding bilirubin's formation and its ultimate function in the human body.

Materials and Methods: A methodical review of bilirubin in humans and animals was carried out and information was gathered using published scientific journals, books and conference proceedings. Articles based on case studies of elevated levels of bilirubin were analysed thoroughly.

Results: Even though, for numerous years, bilirubin was assumed to be merely a desecrate product of the haem catabolic pathway by greatest and a likely lethal compound at worst, statistics from the last few decades clearly show that placidly high serum bilirubin levels are robustly related to have abundant beneficial effects on the human body.

Conclusion: This study reveals new insights into the presence of the only animal pigment found in *Strelitzia nicolia* arils, the potential advantages of bilirubin found in a plant and its therapeutic value indications. This review hopes to resuscitate researchers' credence regarding bilirubin as a toxic compound.

Key words: bilirubin, haem, biliverdin, biliverdin reductase, *Strelitzia nicolai*

Introduction

The catabolism of haem is the only natural progression in human beings that is colorimetric. This process would have been recognized by early man several thousand decades ago. After being injured directly on the skin, a contusion that was black or purple (colours of haem) progressively changed to olive, the colour of biliverdin, and ultimately to yellow, the colour of bilirubin (Otterbein and Choi, 2000). Previously thought to be an "animal-only" pigment in 2009, Florida International University researcher Pirone, (2010) made a breakthrough discovery in finding bilirubin in the arils of *Strelitzia nicolai* Regel and Körn. commonly known as the white bird of paradise. Bilirubin is a by-product from the secondary degradation of haem, the primary being biliverdin. In birds, reptiles and amphibians, biliverdin is the principal end product of haem dilapidation. In mammals, biliverdin endures an added metabolism to form bilirubin. This phenomenon has always mystified scientists.

Formation of Bilirubin in humans

Bilirubin is the terminal product of haem metabolism. Haem is established in haemoglobin, the major component of red blood cells. In humans, 250–400 mg of bilirubin is produced daily, of which more or less 20% is produced from non-haemoglobin sources (London et al., 1950). The conversion of haem to bilirubin is a twostep procedure (Figure 1). Firstly, microsomal haem oxygenase (HO) enzyme catalyses the oxidation of haem to a green tetrapyrrolic bile pigment, biliverdin. Subsequently, biliverdin is transformed to bilirubin by biliverdin reductase (McDonagh, 2001). This reaction can occur in nearly every cell. An illustration is the formation of a bruise that undergoes different colours as it progressively mends i.e. red haem to green biliverdin to yellow bilirubin. Under standard physiological circumstances, the motion of haem oxygenase peaks in the spleen where mature erythrocytes are sequestrated and annihilated.

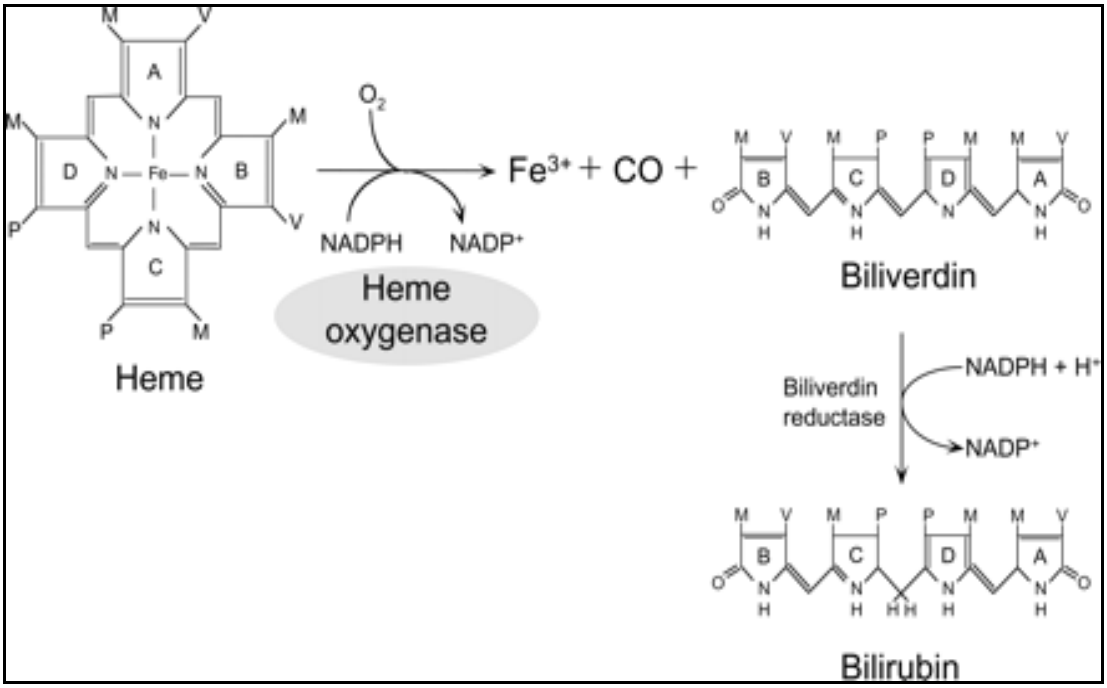


Figure 1: Enzyme-catalysed degradation of haem (Ramadori et al., 2000). Haem degradation starts by haem oxygenase-catalysed oxidation of the *a*-bridge carbon of haem that is converted to CO, directed to aperture of the tetrapyrrole ring which releases the iron molecule. The resulting biliverdin fragment is consequently abridged to bilirubin by cytosolic biliverdin reductase.

Metabolism of Bilirubin

Red blood cells are constantly undergoing a haemolysis course. The typical life-span of a red blood cell is 120 days. Whilst the red blood cells collapse, the haemoglobin is degraded or broken into globin, iron, and haem. The haem is first changed into biliverdin which is quickly reduced to bilirubin. This process occurs in the reticuloendothelial cells of the liver, spleen and bone marrow (Ramadori et al., 2000).

Haemoglobin which is broken down to haem in the spleen is changed to form unconjugated bilirubin (Figure 2). This unconjugated bilirubin is insoluble in water because of its intramolecular hydrogen bonding and attaches to albumin which is then sent to the liver. Inside the liver, bilirubin is conjugated through glucuronic acid by the enzyme glucuronyltransferase, rendering it water soluble. Most of it goes into the small intestine together with the bile. About 95% of the bile created is taken back up by the liver. This bile is then re-secreted by the liver into the small intestine. The procedure is identified as the enterohepatic circulation. About half of the conjugated bilirubin in the large intestine is exercised by gut bacteria to produce urobilinogen. This is then additionally oxidized to urobilin and stercobilin. Urobilin, stercobilin and their disintegrated products give faeces and urine its distinct colour (Kuntz, 2008).

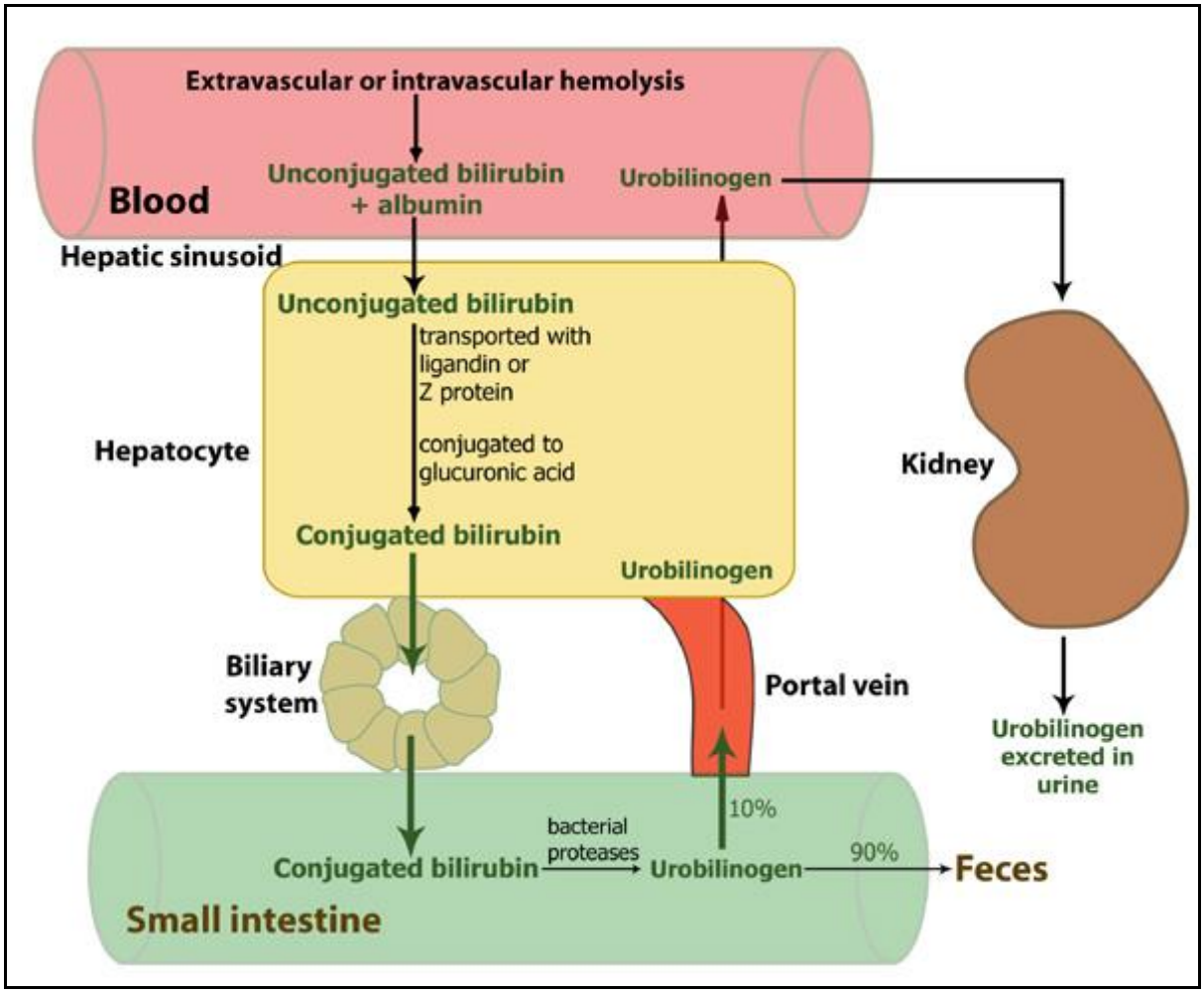


Figure 2: The enterohepatic circulation system (Sedlak and Snyder, 2004). Bilirubin is delivered to the liver, largely attached to albumin. In the liver, bilirubin is conjugated, excreted in the bile, modified in the gastrointestinal tract and largely lost via the faeces.

Bilirubin toxicity

Toxicity resulting from unconjugated hyperbilirubinemia has been known for more than a century since the landmark study of Schmorl (1903) describing autopsy findings from 120 jaundiced infants (Hansen, 2000). Schmorl (1903) contributed immensely to the understanding of epidemiology and pathophysiology of neonatal jaundice and kernicterus.

Biliary efflux is the key route of the removal of bilirubin. Decrease in excretion will lead to the toxicity of intracellular organelles and physiological processes. Amassing of bilirubin and bilirubin

conjugates in human tissues generates jaundice which is distinguished by elevated plasma bilirubin levels and deposition of yellow bilirubin tinctures in skin, sclera, mucous membranes, and other less noticeable tissues (Lathe, 1972). Kernicterus is a form of brain damage caused by excessive jaundice.

The Gilbert syndrome is a harmless hereditary variation connected to unceasingly elevated plasma levels of unconjugated bilirubin (Ritter et al., 1992). This condition emerges when the hepatic expression of the enzyme that conjugates bilirubin, glucuronosyltransferase type 1A1, is reduced. A more lethal inherited disorder affecting the metabolism of bilirubin is called the Crigler–Najjar syndrome. This uncommon disorder is a hereditary form of non-haemolytic jaundice, which is caused by mutations in the UGT1A gene encoding bilirubin glucuronosyltransferase. These produce elevated levels of unconjugated bilirubin which frequently cause brain damage in infants (Ritter et al., 1992).

The Dubin–Johnson disease is an autosomal recessive condition which brings about an addition of conjugated bilirubin in the serum with no increase of liver enzymes (ALT, AST). The above disorder is fabricated by the insufficiency of the canalicular multispecific organic anion transporter (cMOAT) gene (ABCC2/MRP2). This is connected to a flaw in the capability of hepatocytes to secrete conjugated bilirubin into the bile (Paulusma, et al., 1997). Mutations in the *SLCO1B1* and *SLCO1B3* genes cause the Rotor syndrome (non-icteric jaundice). The *SLCO1B1* and *SLCO1B3* genes code for creating proteins, called organic anion transporting polypeptide 1B1 (OATP1B1) and organic anion transporting polypeptide 1B3 (OATP1B3). The above proteins are established in the liver cells. These proteins convey bilirubin, starting from the blood haem to the liver. The *SLCO1B1* and *SLCO1B3* gene alterations that produce the Rotor syndrome lead to strangely small, nonfunctional OATP1B1 and OATP1B3 proteins or a nonexistence of these proteins. Lacking the occupation of these transfer proteins, bilirubin is reducibly taken up by the liver and eliminated from the body (Strassburg, 2010).

Potential beneficial effects of bilirubin

For many years, unconjugated bilirubin (UCB) was considered a useless waste product of haem catabolism with no physiological function but with potential toxicity. If this was a reality, then why would the energy intense conversion of biliverdin to the water insoluble ‘hazardous’ bilirubin which requires additional resources for shipping and secretion occur? The breakthrough of finding the antioxidant and other properties of bilirubin has proposed that this conversion of biliverdin to bilirubin may actually be in reality, a progress in evolution (McDonagh, 2001). On the other hand, Cornelius, (1986) established that the enzyme biliverdin reductase existed even in Cyanobacteria, far prior to the existence of Eukaryotes on earth. Qin, (2007) proposed that the inactivation of digestive

proteases is the evolutionary dynamic strength for bilirubin preponderance. The quantity of digestive proteases secreted by the pancreas largely relies on the quantity of protein that is consumed. This presents a justification for bilirubin's presence, mainly in carnivores or omnivores whereas biliverdin is mostly found in herbivores (Howard and Yudkin, 1963).

Near the beginning of 1950, bilirubin was alleged to guard against the oxidation of lipids, for instance linoleic acid and vitamin A (Bernhard et al., 1954). Interestingly, evidence obtained in the last decade revealed a beneficial role of the molecule. Undeniably, physiological or modestly elevated serum levels of UCB have been shown to have a protective effect in several disorders, paradoxically even including neurodegenerative diseases. Protective effects of UCB rely on its antioxidant properties (Cuadrado and Rojo, 2008). Not only does bilirubin protect against oxidation but it has also been reported to be beneficial against amyotrophic lateral sclerosis (Iłżecka and Stelmasiak, 2003), atopic dermatitis (Tsukahara et al., 1976), cancer (Temme et al., 2001) and coronary artery disease (Hopkins et al., 1996). In diabetes mellitus, Han et al., (2010) discovered that an elevated intensity of serum bilirubin is related to a poorer hazard of the ailment.

Bilirubin as an antioxidant and cytoprotectant

Oxidative stress is damaging to life progression and is mainly accountable for maturation and age-related diseases. Oxidative stress is fundamentally a disproportion among the manufacturing of free radicals and the capability of the cells to counteract their damaging effects. For that reason, generally, the body is capable of producing a range of natural defence mechanisms in opposition to oxidative stress.

Thomas et al., (2008) found that the bilirubin pathway was a physiological cytoprotectant. Bilirubin principally defends against lipid peroxidation. This antioxidant effect impacts cell continued existence as cell loss is additionally clearly amplified following the reduction of bilirubin. Once bilirubin performs as an antioxidant, it is oxidized to biliverdin which is then instantly reduced by biliverdin reductase to bilirubin.

Llesuy and Tomaro (1994) found that an increase in bilirubin production may perhaps be a reaction to early oxidative stress. They discovered that administering CO (II) to rats lead to oxidative stress which precedes haem oxygenase stimulation. The introduction of this enzyme might be a method during the amplification of bilirubin levels, to reduce the harm ignited by oxidative stress.

During the infant period, most of the natural antioxidant level is fairly low compared with bilirubin. Bilirubin which is deadly to neuronal cells at elevated levels has been reported to have cytoprotective

activity at lower concentrations (Breimer et al., 1995). Neonatal jaundice might also have a defensive outcome for the new-born moving for the first moment in time into an unhygienic environment.

An investigation based on fit individuals assembled by low, in-between and elevated serum bilirubin intensity disclosed that high bilirubin levels guard against coronary flow reserve impairment, coronary microvascular dysfunction and, probably, coronary atherosclerosis as well (Gullu et al., 2005).

Biliverdin reductase is an evolutionarily preserved enzyme changing biliverdin to bilirubin, the potent physiological antioxidant. Bilirubin defends cells in opposition to high levels of hydrogen peroxide (H_2O_2), which oxidise bilirubin into biliverdin which is then recycled flipside into bilirubin by biliverdin reductase (Baranano et al., 2002).

Bilirubin as an anti-cancer agent

Cancer is a term used for diseases in which atypical cells multiply without control and are able to invade other tissues. Cancer is a conglomerate of diseases. There are numerous types of cancers that are named according to the organ they affect.

Zucker et al., (2004) researched a vast number of people in the United States and with this they found that the probability proportion for colorectal cancer is condensed to 0.295 in men and 0.186 in women per 1 mg/dL increase in serum bilirubin intensity. Another study in Belgium by Temme et al., (2001) confirmed that there is a contrary connection among serum bilirubin concentration and cancer mortality.

The American Association for Cancer Research (2013) found that a 7.02 occurrence rate of lung cancer per 10,000 person-years for men with bilirubin concentration of 0.68 mg/dL or a smaller amount compared with a prevalence rate of 3.73 amongst men, bilirubin concentrations were 1.12 mg/dL or higher. This interprets into a 51 % increase in the danger for developing lung cancer for patients with reduced bilirubin. Additionally, it was discovered that a lung cancer-specific mortality rate of 4.84 for men with the least amount of bilirubin compared with a mortality rate of 2.46 for men with the highest bilirubin concentration - a 59 % increase in lung cancer-specific death amongst persons with the lowest bilirubin concentration.

Bilirubin as a protectant in diabetes

Diabetes mellitus, the trademark of which consists of higher plasma glucose, is constantly coupled with amplified oxidative stress as well as improved development of complex glycation end products.

Overproduction of oxidizing particles causes the progressive loss of pancreatic β-cells, reducing insulin levels (Yamagishi et al., 2011).

Numerous studies have deduced that elevated bilirubin levels are contrariwise linked to the incidence of type 2 diabetes. Korean scientists reported considerable resistance of hyperbilirubinemic Gunn rats to initiated diabetes, following intraperitoneal contact to streptozotocin compared with their normobilirubinemic littermates (Fu et al., 2010).

Cheriyath et al., (2010) confirmed that bilirubin might contribute a vital responsibility in glycaemic management. Improved expression of haem oxygenase-1, the enzyme responsible for the alteration of haemoglobin to bilirubin, is related with better insulin sensitivity and glucose metabolism. Additionally, serum bilirubin is contrariwise connected to insulin resistance since it amplifies the expression of glucose transporter-1 and the speed of glucose uptake.

Bilirubin as a protectant in cardiovascular disease

Cardiovascular diseases comprise hypertension, atherosclerosis, coronary artery illness and stroke. These are the leading causes of deaths in the world. Schwertner et al., (1994) established that a 50% reduction in total bilirubin was related to a 47% increment in the more rigorous coronary artery diseases.

Gilbert's syndrome is largely one of the frequent inherited genetic ailments. It is caused by an impaired glucuronyl transferase action leading from a slight to modest increase of serum bilirubin. Vitek et al., (2002) reported that the occurrence of ischemic heart condition in Gilbert's syndrome was established to be only 2% in contrast with 12% in control individuals.

Hopkins et al., (1996) established and completed a preceding study that indicates an increase in serum bilirubin level contained by the standard range is linked to a noteworthy and obvious decrease in coronary artery disease risk. Hopkins and co-workers (1996) observed a 60% to 90% decrease in danger once serum bilirubin was in the higher two control quintiles compared with the lowest quintile for both men and women.

Bilirubin as a protector against neurological diseases

Takahashi et al., (2000) proposed that bilirubin is a powerful antioxidant in opposition to the cellular destructive reactive oxygen species and contributes to the overall antioxidant network of the brain.

Bilirubin has been established to be a powerful antioxidant in the brain, acting to forage peroxyl radicals as efficiently as α-tocopherol or vitamin E.

Iwasaki et al., (2005) found that a decreased concentration of serum bilirubin is correlated to an improved danger of neurodegenerative diseases. In addition, a low concentration of serum bilirubin is related to an increased risk of amyotrophic lateral sclerosis and psychological disorders such as winter depression and schizophrenia.

The breakthrough of the only animal pigment (bilirubin) found in a plant

Strelitziaceae is a tropical monocotyledonous family generating colourful bracteate inflorescences with woody capsular fruits that contain vibrantly coloured arillate seeds. Pirone, (2010) discovered in the arils of *S. nicolai* (the White Bird of Paradise) and *Strelitzia reginaee* (the bird of paradise), the presence of bilirubin. The seeds contain an inner and outer integument, micropylar zone and an appendage in the form of dense tufts of a bright orange coloured aril (Van de Venter, 1986). Bilirubin was present in these arils as the primary pigment and thus functions to produce vivid colours to attract animals.

Not like general plant pigments that decompose once the cell dies, aril pigments in the family endure for decades. Pirone, (2010) isolated the orange pigment from the arils of *S. nicolai* and performed HPLC-ESMS, UV-visible, H NMR and C NMR investigation to conclude its chemical structure. The chemical properties of the compounds were atypical and did not equal those of known colour classes (carotenoids, flavonoids, betalains and the chlorophylls). The results revealed that the pigment was bilirubin-IX, an orange-yellow tetrapyrrole until that time known only in mammals and some other vertebrates.

The incidence of bilirubin discovery was not limited to *S. nicolai*. Two other species in the *Strelitziaceae*, *Phenakospermum guyannense* A.Rich. and *S. reginaee* contain aril pigments which co-eluted with genuine bilirubin in HPLC and had similar UV-visible spectra (Pirone, 2010). In the arils of the *Strelitzia* species, bilirubin was present as the principal pigment and thus functioned to create colour. Previously, no tetrapyrroles were known to generate display colour in plants (Pirone, 2010). Bilirubin is therefore the initial creation of a further biosynthetic course, the tetrapyrrole pathway, to produce eye-catching colour in a plant reproductive constitution.

Tetrapyrroles are organic particles that had great impact over the progression of existence for more than 4 billion years. These molecules play imperative roles in a variety of natural processes, including

photosynthesis and respiration. Higher plants hold four classes of tetrapyrroles, namely, chlorophyll, haem, sirohaem and phytochromobilin. Every one of the tetrapyrroles is derived from an ordinary biosynthetic pathway (McDonagh, 2001). The chief location of tetrapyrrole biosynthesis in plants are in the plastids. Tetrapyrrole biosynthesis is dominantly regulated by acclimation to environmental conditions.

Pathway of tetrapyrrole biosynthesis

The initial phase in the tetrapyrrole biosynthesis is the production of 5-aminolaevulinic acid ALA via two probable routes: (a) condensation of succinyl CoA and glycine (C4 pathway) using ALA synthase or (b) decarboxylation of glutamate (C5 pathway) via three diverse enzymes, glutamyl-tRNA synthetase to charge a tRNA with glutamate, glutamyl-tRNA reductase to decrease glutamyl-tRNA to glutamate-1-semialdehyde (GSA) and GSA aminotransferase to catalyse a transamination response to manufacture ALA (Grimm, 2003).

The next step is the conversion of ALA to uroporphyrinogen III. This is attained by the achievement of three enzymes in a single general pathway: porphobilinogen (PBG) synthase or ALA dehydratase to condense two ALA fragments to produce porphobilinogen, hydroxymethylbilane synthetase, to polymerise four PBG molecules into preuroporphyrinogen (tetrapyrrole structure) and uroporphyrinogen III synthetase to connect two pyrrole components as one to yield uroporphyrinogen III (Grimm, 2003). To manufacture haem and chlorophyll, uroporphyrinogen III requires being decarboxylated into coproporphyrinogen III by the act of uroporphyrinogen III decarboxylase (as illustrated in Figure 3).

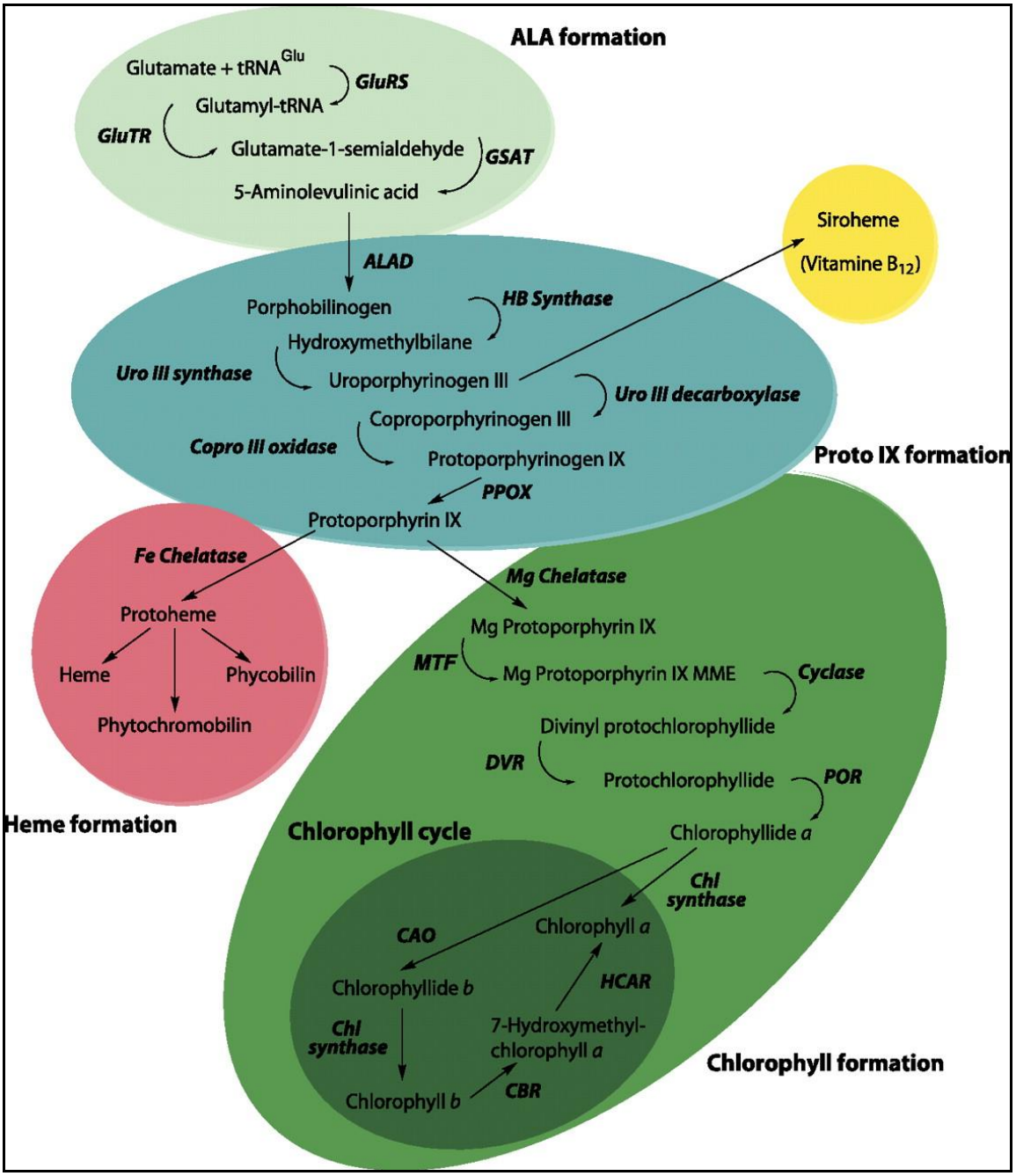


Figure 3: Biochemical pathway of plant tetrapyrrole biosynthesis. The global precursor of all tetrapyrroles, 5-aminolevulinic acid (ALA), is produced from glutamate. ALA is additionally processed to protoporphyrin IX before the pathway branches into haem and chlorophyll biosynthesis (Czarnecki and Grimm, 2012).

Differences and similarities of the tetrapyrrole metabolic synthesis in animals and plants

The enzymatic steps are the most important differences in the pathways. Another major difference is the two routes of ALA synthesis, which developed independently during evolution (Tanaka and Tanaka, 2007). These two paths differ in the commencing metabolites. The earliest path is found in all bacteria and plants. It starts with glutamate and three enzymes needed to form ALA. In animals and yeasts, a succinyl-CoA-glycin entry was elucidated with one enzyme to form ALA (Mochizuki, 2010).

The configuration of haem in all organisms is attained by the chronological act of eight discernible enzymes. The plant tetrapyrrolic pathway additionally includes several bicurations for the synthesis of different products (Tanaka and Tanaka, 2011).

A major distinction of plant and animal tetrapyrrolic biosynthesis pathway is the different subcellular localization of the pathway. The animal metabolic chain is spatially separated within the cell (Grimm, 2003). Four enzymes are located in the cytoplasm and another four are present in the mitochondria. In plants, the whole metabolic pathway for chlorophyll is located in the chloroplasts. Here, the synthesis takes place in plastids and in the mitochondria (Jensen et al., 1999).

Can bilirubin's newfound status be harnessed therapeutically?

Despite a century of research, several clinical areas of bilirubin remain poorly understood, controversial or unrecognized. The breakthrough in finding profuse amounts of bilirubin in the deep orange turfed arils of the seeds of the *Strelitzia sp.* and *P. guyannense* has opened up new opportunities for the possible insight into the evolutionary reason for the baffling biosynthesis in humans.

It has been acknowledged that tropical rainforest environments are so nutrient poor that humans could not survive within or without access to food crops. Recent investigations into the diets of Amazonian

forest dwellers have discovered that their diets consist primarily of palm nut and *P. guyannense* seeds. The seeds are ground, powdered and made into porridge. Arils are also chewed and the juices are consumed. Ambiguously, these forest dwellers live very extended and healthy lives (Milton et al, 1991). Could it be due to the bilirubin found in the arils that forms part of their primary diet? If so, this could be the cytoprotective effect suspected to be the underlying characteristic of bilirubin.

Sunbirds are the main pollinators of the 'white bird of paradise tree'. Birds do not produce bilirubin. They produce biliverdin. Therefore, this could be the reason they consume the bilirubin found in the arils. In fact, an elevated level of bilirubin in birds, after consumption, does not impair them in any way. Therefore, there is rationale to believe that bilirubin found in these arils could have a possible protective effect.

The human body is a multifaceted being which has the aptitude to mend itself if listened to fittingly. However, in the modern world the variables are almost infinite. It is a fact that people of the world today are having more problems with their health than ever before. It is for this reason that scientists nowadays are looking at our pasts in order to produce medicines for the future since for hundreds of years man has used plant extracts to cure ailments. This study makes us believe that there is now strong experimental and theoretical support for the claim that bilirubin has a host of beneficial effects.

The most important piece of evidence on the subject of bilirubin as a possible beneficial product lie in the fact that in most animal cells the degradation of haemoglobin, if stopped an action prior with a green, soluble fragment called biliverdin, the desecrate would be simply emitted with no danger of detrimental accumulation. However, as an alternative to stopping at biliverdin, for the most part, animal cells persist to formulate bilirubin by means of biliverdin reductase. If the only result of bilirubin in high amounts is toxicity, then it is illogical that animals would produce it using a high energy-consuming step.

Conclusion

In this review, the investigational as well as clinical studies on the relationships between bilirubin and its protective effect on certain aspects of the human body were conferred. These studies collectively illustrate that low serum bilirubin levels are linked with an increased risk of several pathological conditions whereas mildly elevated serum bilirubin levels provide protection. It is for this reason we can therefore hypothesize that if the bilirubin found in the human body has a protective effect against numerous conditions, perhaps the bilirubin found in the arils of *S. nicolai* will have similar effects and could be harnessed therapeutically to modulate certain pathological conditions. Clearly, a detailed study to investigate this potential further emerges necessary and will unquestionably provide much needed substantiation.

References

1. American Association for Cancer Research (AACR) (2013). Novel serum biomarker bilirubin predicted lung cancer risk in smokers.
2. Baranano, D.E., Rao, M. and Ferris, C.D. (2002). Snyder S.H. Biliverdin reductase: a major physiologic cytoprotectant. *Proc. Natl. Acad. Sci. U.S.A.* 99: 16093–16098.
3. Bernhard, K., Ritzel, G. and Steiner, K.U. (1954). On a biological significance of bile pigments: bilirubin and biliverdin as antioxidants for vitamin A and essential fatty acids. *Helv Chim Acta*. 37: 306–31.
4. Breimer, L.H., Wannamethee, G. and Ebrahim, S. (1995). Serum bilirubin and risk of ischemic heart disease in middle-aged British men. *Clin Chem*. 41: 1504–1508.
5. Cheriyath, P., Gorrepati, V.S., Peters, I., Nookala, V., Murphy, M.E, Srouji, N. and Fischman, D. (2010). High total bilirubin as a protective factor for diabetes mellitus: An analysis of NHANES data from 1999-2006. *J Clin Med Res*. 2: 201–206.
6. Cornelius, C.E. (1986). Comparative bile pigment metabolism in vertebrates. In: Ostrow JD, 601–647.
7. Cuadrado, A. and Rojo, A.I. (2008). Heme oxygenase-1 as a therapeutic target in neurodegenerative diseases and brain infections. *Curr Pharm Des*. 14: 429-442.
8. Czarnecki, O. and Grimm, B. (2012). Post-translational control of tetrapyrrole biosynthesis in plants, algae, and cyanobacteria. *J. Exp. Bot*. 63 (4): 1675-1687.
9. Fang, L.S and Bada, J.L. (1990). The blue-green blood plasma of marine fish, Clinocottus analis, with green blood plasma. *J.fish Biol* 33: 775-780.
10. Fu, Y.Y., Kang, K.J., Ahn, J.M., Kim, H.R., Na, K.Y., Chae, D.W., Kim, S. and Chin, H.J. (2010). Hyperbilirubinemia reduces the streptozotocin-induced pancreatic damage through attenuating the oxidative stress in the Gunn rat. *Exp. Med*. 222: 265–273.
11. Grimm B. (2003). Regulatory mechanisms of eukaryotic tetrapyrrole biosynthesis. The porphyrin handbook: iron and cobalt pigment, biosynthesis, structure and degradation. Volume 12: 1-2.
12. Gullu, H., Erdogan, D., Tok, D., Topcu, S., Caliskan, M., Ulus, T. (2005). High serum bilirubin concentrations preserve coronary flow reserve and coronary microvascular functions. *Arteriosclerosis, Thrombosis and Vascular Biology*. 25: 2289–2294.
13. Han, S.S., Na, K.Y., Chae, D.W., Kim, Y.S., Kim, S. and Chin, H.J. (2010). High serum bilirubin is associated with the reduced risk of diabetes mellitus and diabetic nephropathy. Tohoku J. *Exp. Med.* 221: 133–140.
14. Hansen, T.W.R. (2000). Pioneers in the scientific study of neonatal jaundice and kernicterus. *Pediatrics*. 106: 2-15.

15. Hopkins, P.N., Wu, L.L., Hunt, S.C., James, B.C., Vincent, M.G. and Williams, R.R. (1996). Higher Serum Bilirubin Is Associated With Decreased Risk for Early Familial Coronary Artery Disease. *Arterioscler Thromb Vasc Biol*. 16(2): 250-5.
16. Howard, F. and Yudkin, J. (1963). Effects of dietary change upon the amylase and trypsin activities of the rat pancreas. *Brit. J. Nutr*. 17: 28-37.
17. Iłżecka, J. and Stelmasiak, Z. (2003). Serum bilirubin concentration in patients with amyotrophic lateral sclerosis. *Elsevier*. 8: 20-954.
18. Iwasaki, Y., Igarashi, O., Iwasa, Y., Hirano, K., Satoh, R., Iwamoto, K., Kawase, Y., Aoyagi, J., Ichikawa, Y., Kawabe, K. (2005). Bilirubin and amyotrophic lateral sclerosis. *Clin. Neurol. Neurosurg*. 5: 107- 160.
19. Jensen, P.E., Gibson, L.C. and Hunter, C.N. (1999). ATPase activity associated with the magnesium-protoporphyrin IX chelatase enzyme of Synechocystis PCC6803: evidence for ATP hydrolysis during Mg2+ insertion, and the MgATP-dependent interaction of the ChlI and ChlD subunits. *Biochem. J*. 339: 127–134.
20. Kuntz, E. (2008). Hepatology: Textbook and Atlas. Germany: Springer. 38, ISBN 978-3-540-76838-8.
21. Lathe, G.H. (1972). The degradation of haem by mammals and its excretion as conjugated bilirubin. *Essays Biochem*. 8: 107-148.
22. Llesuy, S.F. and Tomaro, M.L (1994). Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. *Biochimica et Biophysica Acta*. 1223: 9-14.
23. London, I.M., West, R. and Shemn, D. (1950). On the origin of bile pigment in man. *J Biol Chem*. 184: 351–358.
24. McDonagh, A.F. (2001). Turning green to gold. *Nat Struct Biol*. 200: 98–200.
25. Milton, K., Knight, C.D. and Crowe, I. (1991). Comparative aspects of diet of Amazonian forest dwellers. Phil. Trans. 334: 253-263.
26. Mochizuki, N., Tanaka, R., Grimm, B., Masuda, T., Moulin, M. and Smith, A.G. (2010). The cell biology of tetrapyrroles: a life and death struggle. *Trends Plant Sci*. 15: 488–498.
27. Otterbein, L.E and Choi, A.M. (2000). Heme oxygenase: colors of defense against cellular stress. *J Physiol Lung Cell Mol Physiol.* 279(6): 29-37.
28. Paulusma, C.C., Kool, M. and Bosma, P.J. (1997). A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin–Johnson syndrome. *Hepatology*. 25: 1539–1540.
29. Pirone C. (2010). "Bilirubin: an Animal Pigment in the Zingiberales and Diverse Angiosperm Orders". FIU Electronic Theses and Dissertations. Paper 336. http://digitalcommons.fiu.edu/etd/336.

30. Qin, X. (2007). Inactivation of digestive proteases by deconjugated bilirubin: the possible evolutionary driving force for bilirubin or biliverdin predominance. *Gut.* 56(11): 1641–1642.
31. Ramadori, G., Immenschuh, S. and Medizin, I.Z. (2000). Gene regulation of heam oxygenase-1 as a therapeutic target. *Abteilung Gastroenterologie und Endokrinologie*. 48: 53—57.
32. Ritter, J.K., Yeatman, M. T., Ferreira, P. and Owens, I.S. (1992). Identification of a genetic alteration in the code for bilirubin UDP- Glucuronosyltransferase in the UGT1 gene complex of a Criler-Najjar type 1 patient. *J Clin Invest*. 90: 150-155.
33. Schwertner, H.A., Jackson, W.G. and Tolan, G. (1994). Association of low serum concentration of bilirubin with increased risk of coronary artery disease. *Clin. Chem.* 40: 18–23.
34. Schmorl G. (1903). Zur Kenntnis des ikterus neonaturum, insbesondere der dabei auftretenden gehirnveranderungen.*Verh Dtsch Ges Pathol*. 6 :109–15.
35. Sedlak, T.W., Snyder, S.H. (2004). Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics* 113: 1776-82.
36. Strassburg, C.P. (2010) Hyperbilirubinemia syndromes (Gilbert-Meulengracht, Crigler-Najjar, Dubin-Johnson, and Rotor syndrome). *Clin Gastroenterol*. 5, 555-571.
37. Takahashi, M., Dore, S., Ferris, C.D., Tomita, T., Sawa, A., Wolosker, H., Borchelt, D.R., Iwatsubo, T., Kim, S.H., Thinakaran, G., Sisodia, S.S. and Snyder, S.H. (2000). Amyloid precursor proteins inhibit heam oxygenase activity and augment neurotoxicity in Alzheimer's disease. *Neuron.* 28: 461–473.
38. Tanaka, R. and Tanaka, A. (2011). Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes. *Biochim. Biophys. Acta*. 180 : 968–976
39. Tanaka, R. and Tanaka, A. (2007). Tetrapyrrole biosynthesis in higher plants. *Annual Review of Plant Biology*. 58: 321–346.
40. Temme, H.M.E., Zhang, J., Schouten, G.E. and Kesteloot, H. (2001). Serum bilirubin and 10-year mortality risk in a Belgian population. *Cancer Causes and Control*. 12: 887-894.
41. Thomas, W., Sedlaka, M.S., Higginsonb, D.S., Paulb, B., Julurib, K.R. and Snydera, S.H. (2008). Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. *Pubmed*. PMCID: PMC266404.
42. Tsukahara, H., Shibata, R., Ohshima, Y., Sato, S., Ohta, N., Hiraoka, A., Nishima, S. and Van de Venter, H.A. (1976). A note on ovular outgrowths in the species of *Strelitzia*. *J. S Afr Bo* 42: 37-39.
43. Van de Venter, H.A. (1986). Some characteristics of leaf and inflorescence production in *Strelitzia* reginae. *J. S Afr Bo*. 4: 305-31.

44. Vitek, L., Jirsa, M., Brodanova, M., Kalab, M., Marecek, Z., Danzig, V., Novotny, L., Kotal, P. (2002). Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels . *Atherosclerosis*. 160: 449–456.
45. Yamagishi, S.I., Maeda, S., Matsui, T. and Ueda, S. (2011). Fukami K and Okuda S. Role of advanced glycation end products (AGEs) and oxidative stress in vascular complications in diabetes. *Biochim. Biophys. Acta*. 20-27.
46. Zucker, S.D., Horn, P.S. and Serman, K.E. (2004). Serum bilirubin levels in the U.S. population: gender effect and inverse correlation with colorectal cancer. *Hepatology*. 40: 827–835.

Chapter 3

Journal article for publication 2:

In vitro chemo-preventative activity of *Strelitzia nicolai* aril extract containing bilirubin

Depika Dwarka[1*], Veneesha Thaver[2], Mickey Naidu[3], Neil A. Koorbanally[4] and Himansu Baijnath[5]

[1,5]School of Biological Sciences, University of KwaZulu- Natal, Westville Campus, Durban. South Africa; [2]School of Laboratory Medicine and Medical sciences, Discipline of Physiology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa; [3]School of Health Sciences, Discipline of Pharmacology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa; [4]School of Chemistry and Physics, University of KwaZulu-Natal, Westville Campus, Durban. South Africa.

***Email:** depika.dwarka@gmail.com

Abstract

Background: The discovery of the only animal pigment, bilirubin, in the plant *Strelitzia nicolai* Regel and Körn has triggered a vast number of questions regarding bilirubin's formation and its role in the human body. Recent studies have confirmed that bilirubin, at certain levels, has many medical benefits. Various case studies have revealed that bilirubin is a potent antioxidant. Cervical cancer is one of South Africa's largest women's health crises. It is estimated that it affects one out of 41 South African women and kills approximately 8 women in the country every day. Thus, the aim of this study was to investigate if the aril extract of *S. nicolai* containing bilirubin possesses anti-cancer activity and to determine its effect on the induction of apoptosis. **Materials and methods:** The 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) activity was firstly used to determine the antioxidant effect of the extract. Thereafter, the cytotoxic effect was tested using the 2, 3-Bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay. Apoptosis was confirmed and quantified using the

Annexin V-PE kit and cellular morphology was studied using acridine orange and ethidium bromide. **Results:** The aril extract decreased cell viability by 52% and induced apoptosis in HeLa cells, as shown by the Annexin V-PE Apoptosis detection kit and morphological studies with acridine orange/ethidium bromide staining. **Conclusion:** The activity of the extract as a potent antioxidant was immensely enhanced, as compared to the bilirubin standard. These results suggest that *S. nicolai* aril extract containing bilirubin works synergistically as opposed to bilirubin on its own. Furthermore, this extract might be a good candidate for the therapeutic intervention of cervical cancer.

Keywords: Bilirubin, *Strelitzia nicolai,* apoptosis, aril extract, antioxidant

Introduction

Bilirubin is an end product from the breakdown of haemoglobin molecules in red blood cells. Firstly, microsomal haem oxygenase (HO) enzyme catalyses oxidation of haem to green tetrapyrrolic bile pigment, biliverdin. Biliverdin is subsequently transformed to bilirubin by biliverdin reductase (McDonagh, 2001) and excreted from the body via the bile ducts of the liver, typically as the main component of bile. If bilirubin is not excreted, it leads to the toxicity of intracellular organelles and physiological processes. A build-up of bilirubin leads to jaundice. Jaundice could result from three distinctive processes, i.e. (a) increased production of bilirubin, (b) decreased excretion by the liver and/or (c) bile duct obstruction. The deposition of bilirubin in the connective tissues (skin, scleras, and internal organs) results in a yellow colour, typical of jaundice. The deposition in parenchymal cells (in basal ganglia) is called kernicterus (intracellular accumulation pigments). This is a rare condition caused by increased levels of unconjugated bilirubin that is lipid-soluble and capable of crossing the blood-brain barrier. Other harmful effects of bilirubin toxicity include the Gilbert syndrome, the Dubin–Johnson syndrome and the Crigler–Najjar syndrome (Lathe, 1972). *Strelitziaceae,* is a tropical monocotyledonous family generating colourful bracteates inflorescences with woody capsular fruits that contain vibrantly coloured arillate seeds. Pirone, (2010) discovered bilirubin in the arils of the white bird of paradise tree (*S. nicolai*) and the bird of paradise (*S. reginae*) being present as the primary pigment, producing colour.

Even though bilirubin was assumed to be only a waste product for decades, recent advances depict that bilirubin serves as a potent antioxidant and anti-cancer agent (Temme et al., 2001). Thomas et al., (2008) found that the bilirubin pathway was a physiological cytoprotectant. Bilirubin, largely protects

against lipid peroxidation. This antioxidant effect impacts cell survival as cell death is more markedly augmented following depletion of bilirubin. When bilirubin acts as an antioxidant, it is oxidized to biliverdin, which is immediately reduced by biliverdin reductase to bilirubin. Llesuy and Tomaro, (1994) found that an increase in bilirubin could be a response to initial oxidative stress. They discovered that administering Co (II) to rats leads to oxidative stress which precedes haem oxygenase induction. The induction of this enzyme could be a mechanism through the increase of bilirubin levels, to decrease the damage caused by oxidative stress.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) participate in processes contributing to cancer progression but also play important roles in endogenous defences, working to eliminate and control the spread of transformed cells. Due to recent increases in the number of cancer patients using antioxidant supplements, a greater understanding of both the damaging and protective actions of ROS in carcinogenesis is crucial to further advances in cancer treatment. Therefore, scientists consider a good antioxidant as also a good anti-cancer agent (Monks et al., 2004).

Medicinal plants contain immunomodulatory and antioxidant properties, leading to anti-cancer activities. They have flexible immunomodulatory activity by invigorating both non-specific and specific immunity (Pandey and Madhuri, 2006). Certain phytochemicals from plants hold strong antioxidant capacity. The antioxidants may prevent and cure cancer and other diseases by protecting the cells from damage caused by free radicals. The body formulates a portion of the internal antioxidants it uses to counteract free radicals. These antioxidants are called endogenous antioxidants. However, the body relies on external (exogenous) sources to acquire the rest of the antioxidants needed (Diplock et al., 1998).

Zucker et al., (2004) investigated a large number of subjects in the United States and with this they found that the odds ratio for colorectal cancer is reduced to 0.295 in men and 0.186 in women per 1 mg/dL increment in serum bilirubin levels. An additional study in Belgium by Temme et al., (2001) showed an inverse relationship between serum bilirubin levels and cancer mortality.

In 2013, the American Association for cancer research found that a 7.02 incidence rate of lung cancer per 10,000 person-years for men with bilirubin levels of 0.68 mg/dL or less compared with an incidence rate of 3.73 among men whose bilirubin levels were 1.12 mg/dL or more. This translates

into a 51 percent increase in the risk of developing lung cancer in patients with low bilirubin (American Association for Cancer Research, 2013).

This newfound evidence gives insight into the reason as to why mammals evolved an energetically expensive and apparently unnecessary enzymatic step for converting the relatively innocuous biliverdin to the more toxic bilirubin.

Recently, our understanding of cancer has advanced in the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype. It is now clear that some oncogenic mutations disrupt apoptosis, leading to tumour initiation, progression or metastasis. Most cytotoxic anti-cancer agents induce apoptosis, increasing the possibility that defects in apoptotic programs contribute to treatment failure. The very same mutations that suppress apoptosis during tumour development also reduce treatment sensitivity. Apoptosis provides a conceptual framework to link cancer genetics with cancer therapy. This, together with the discovery of the only animal pigment in a plant (bilirubin), has led us to the present study that investigates whether the aril extract that contains bilirubin is a potential antioxidant and induces apoptosis in HeLa cell lines. This study also investigated the bilirubin standard that is available commercially and its effects were compared with the aril extract. Many conventional drugs or their precursors are derived from plants. There are fundamental differences between administering a pure chemical and the same chemical in a plant matrix (Murthy et al., 2002).

Materials and methods

Collection of plant material.

Fresh specimens (± 1 kg fresh weight) of *S. nicolai* (Voucher specimen: Dwarka and Baijnath 1 Ward Herbarium UDW) arils were collected from Kwa-Zulu Natal (29.8178° S, 30.9434° E), South Africa during December 2013. The plants were identified using taxonomic keys.

Extraction of bilirubin from arils

Tissue (50 g) from the arils of the plant was ground by a mortar and pestle, extracted with 750 ml of chloroform for 72 hours and filtered through a Buchner funnel. The solution was re-extracted with

50 ml of chloroform, and the procedure repeated until the arils were colourless. The arils were then subsequently air-dried to form a powdery residue which was dissolved in appropriate solvents, depending on the assay.

Preparative thin layer chromatography (TLC) to confirm the presence of bilirubin

TLC was carried out with a commercial standard with a mobile phase of chloroform: methanol: acetic acid (97:2:1). The crude aril extract and bilirubin standard were applied as dots 1 cm from the bottom of the plate. The plate was immersed into the development chamber containing the mobile phase and allowed to develop in the saturated chromatography chamber. Thereafter, the plate was removed from the chamber, allowed to air dry and the Rf value calculated.

Nuclear magnetic resonance (NMR)

The aril extract of *S. nicolai* and bilirubin standard were analysed on a Bruker Advance 500 MHz NMR instrument. 10 mg of the sample was dissolved in 0.5 ml $CDCl_3$ and the ^{1}H and ^{13}C NMR spectra recorded and referenced to the deuterio chloroform signal at δ 7.24 relative to the internal standard TMS. Chemical shifts were expressed in ppm (Jiang et al., 2004).

Measurement of free radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The scavenging activity (antioxidant capacity) of the plant phytocompounds on the stable radical DPPH was evaluated according to the method by Murthy et al., (2002) with some modifications. A volume of 150 µl of methanolic solution of the compound at different concentrations (500, 250, 125, 62.5, 31.3 and 15.6 µg/ml) was mixed with 2.85 ml of a methanolic solution of DPPH (0.1 mM). An equal amount of MeOH and DPPH without sample served as a control. After 30 min of reaction at room temperature in the dark, the absorbance was measured at 517 nm against methanol as a blank using a UV spectrophotometer. The percentage-free radical scavenging activity was calculated according to the following equation: % Scavenging activity = Ac-As/Ac × 100. Where Ac = Absorbance of control and As = Absorbance of sample.

Cell culture

The HeLa (human cervical cancer cell line) was donated by Ms Joubert from the department of infectious diseases, Nelson Mandela School of Medicine, University of KwaZulu Natal. Cells were grown at 37°C in a humidified incubator under 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with foetal calf serum (10 %) and antibiotics (penicillin: 10 000 U/ml,

streptomycin sulphate: 10 000 U/ml). Culture medium was replaced every two days. After confluency, the cells were trypsinized and sub cultured.

Cell viability using 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT assay)

The XTT cytotoxicity assay was conducted according to Jiang et al., (2004) with minor modifications. The assay was carried out in flat-bottomed micro titre plates (96 well) (Cellstar, Greiner, Germany). Cells (50 µl; ± 1.2 $x10^3$) were added into each well and aril extract (50 µl) in a twofold dilution factor (1000µg/ml – 7,8 µg/ml) and DMEM (50 µl) were added to the respective wells. The same procedure was implemented for the bilirubin standard purchased from Sigma Chemicals. In the control wells, cells (50 µl) and Taxol® (250 µg/ml) were added, respectively. The plates were incubated (37°C) in a humidified incubator (5 % CO_2 atmosphere) for 24, 48 and 72 hours. Thereafter, XTT (20 µl) reagent was added and the plates further incubated (4 h; 37ºC) in a humidified incubator (5 % CO_2 atmosphere). The absorbance was read (578 nm) on an ELISA plate reader (Digital Analogue Systems, Italy) and the percentage viability determined using the formula:

% Cell viability = Absorbance of treated cells/Absorbance of untreated cells × 100

Confirmation and quantification of apoptosis

The Annexin V-PE Apoptosis detection kit (BD Biosciences) was used as per the manufacturer's protocol. The HeLa cells were seeded in plates (24 well) and left to adhere overnight. They were then treated with the aril extract of *S. nicolai* (250 µg/ml), bilirubin standard (7. 8 µg/ml) and Taxol® (250 µg/ml). After 24 hours, the cells were trypsinized, washed twice with PBS and re-suspended in binding buffer (1 X at 50 000 cells/ml). FITC Annexin V (5 µl) and propidium iodide (PI) (5 µl) were added to the sample, vortexed and incubated (15 min) in the absence of light. Thereafter, binding buffer (400 µl) was added to each tube and the results analysed by flow cytometry (BDAccuri C6).

Assessment of morphology in apoptosis

The membrane changes of apoptosis were observed using acridine orange and ethidium bromide. A stock solution (100 X) was made up by adding ethidium bromide (50 mg) and acridine orange (15 mg). This was dissolved in ethanol (1 ml; 95 %) and distilled water (49 ml). This was then mixed well

and divided into aliquots (1 ml). The working stock solution (1 X) was made up of an aliquot (1 ml) of the 100 X stock solution and diluted with PBS (99 ml).

A sterile cover slip was inserted into the 24 well plates using sterile forceps. The HeLa cells were seeded in plates and left to adhere overnight. They were then treated with the aril extract of *S. nicolai* (250 µg/ml), bilirubin standard (7.8µg/ml) and Taxol® (250 µg/ml). After 24 hours, the DMEM was removed and 1ml of acridine orange-ethidium bromide solution was added to each well. Thereafter, the cover slips were removed and mounted on a slide and viewed using a fluorescence microscope set for fluorescein (495 nm primary filter and a 515 nm secondary filter). Cells were viewed under the 10X objective where viable normal cells stained bright green with intact structured nuclei and viable apoptotic cells stained green with highly condensed or fragmented nuclei. Non-viable normal cells were identified as those with chromatin stained bright orange and with an organized structure whilst non-viable with apoptotic nuclei were observed to have highly condensed and fragmented chromatin.

Data analysis

Data was analysed using the Graphpad prism 5 for individual comparisons and data was expressed as the mean ± standard deviation. P values less than 0.05 were considered to be statistically significant.

Results

TLC profile of compounds in the arils of *S. nicolai*

The TLC profile revealed two major compounds (B and C) present in the aril extract. The R*f* value of compound B matched that of the bilirubin standard (0.93 in chloroform: methanol: acetic acid (97: 2: 1)). This indicated the presence of bilirubin in the aril extract of *S. nicolai*.

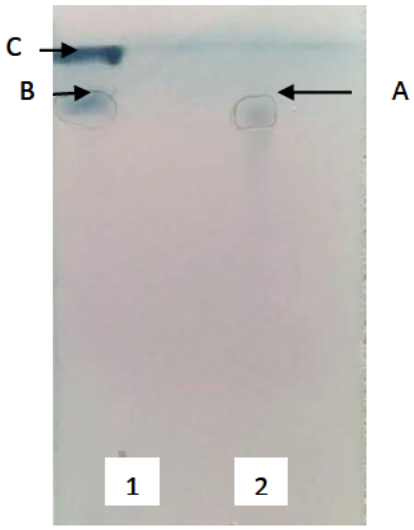


Figure 1: TLC of the chloroform extract of *S. nicolai* arils using chloroform: methanol: acetic acid (97: 2: 1) as the mobile phase and developed with *p*-anisaldehyde spray reagent. Spot 1 is the aril extract and spot 2 is the bilirubin standard.

NMR

The ^{1}H NMR spectrum (refer to Appendix 1) of the aril extract showed similar resonances to that of the bilirubin standard, confirming its presence as one of the major compounds in the aril extract. In essence, the methyl, methylene and ABX coupled protons of the olefinic groups could be identified

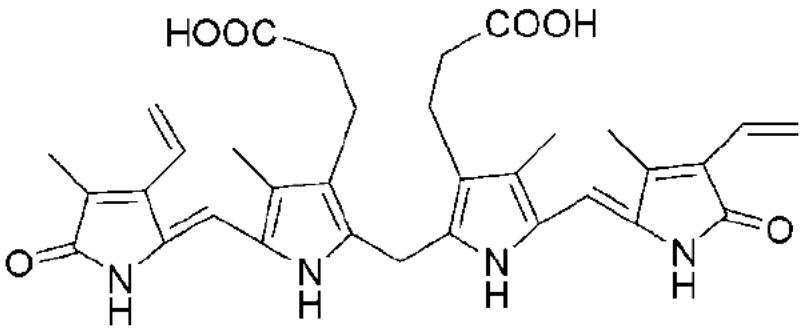


Figure 2: Bilirubin-IXα demonstrating intramolecular H-bonding

Table 1: ^{1}H NMR data of aril extract and bilirubin standard (values are given in ppm)

Proton group	***S. nicolai* aril extract**	**Multiplicity**	**Bilirubin standard**
2-CH_3	1.63	Singlet	1.29
7-CH_3	2.08	Singlet	2.00
13-CH_3	2.08	Singlet	2.06
17-CH_3	2.83	Singlet	2.12
8 & 12-$CH_2CH_2CO_2H$	2.32	Triplet	2.87
8 & 12-$CH_2CH_2CO_2H$	2.08	Triplet	1.57
10-CH_2	4.16	Singlet	4.00
18-CH=CH_2*	~5.30 (centroid)*	ABX system	5.36
18-C*H*=CH_2*	~6.22 (centroid)*	ABX system	6.23
18-CH=CH_2*	~6.6 (centroid)*	ABX system	6.59
3-CH=C*H*$_2$*	~5.63 (centroid)*	ABX system	5.61
3-CH=CH_2*	~5.66 (centroid)*	ABX system	5.64
3-CH=CH_2*	~6.57 (centroid)*	ABX system	6.67
5-H	6.24	Singlet	6.20
15-H	6.15	Singlet	6.15
21-H	10.25	Broad singlet	10.56
22-H	10.83	Broad singlet	10.81
23-H	10.94	Broad singlet	10.70
24-H	9.33	Broad singlet	9.31
COOH	12	Broad singlet	13.73

Antioxidant activity

The DPPH radical scavenging activity revealed that the aril extract had a greater percentage radical scavenging activity than the bilirubin standard. The highest activity was considered at a concentration of 500 µg/ml generating a scavenging activity of 70.9%. Bilirubin produced the highest radical scavenging activity at 250 µg/ml with 52.6%. These results were analysed using graphpad prism 5. The One Way Anova test confirmed that the aril extract and bilirubin standard at each different concentration when compared with each other and then to the positive control ascorbic acid showed no significant difference. This validated that the treatments act in a similar manner to the positive control. The Tukey's multiple comparison test validated that aril vs bilirubin was not significant. The aril vs ascorbic acid was significantly different (*) and bilirubin vs ascorbic acid revealed that they were, to a great extent, significantly different (**).

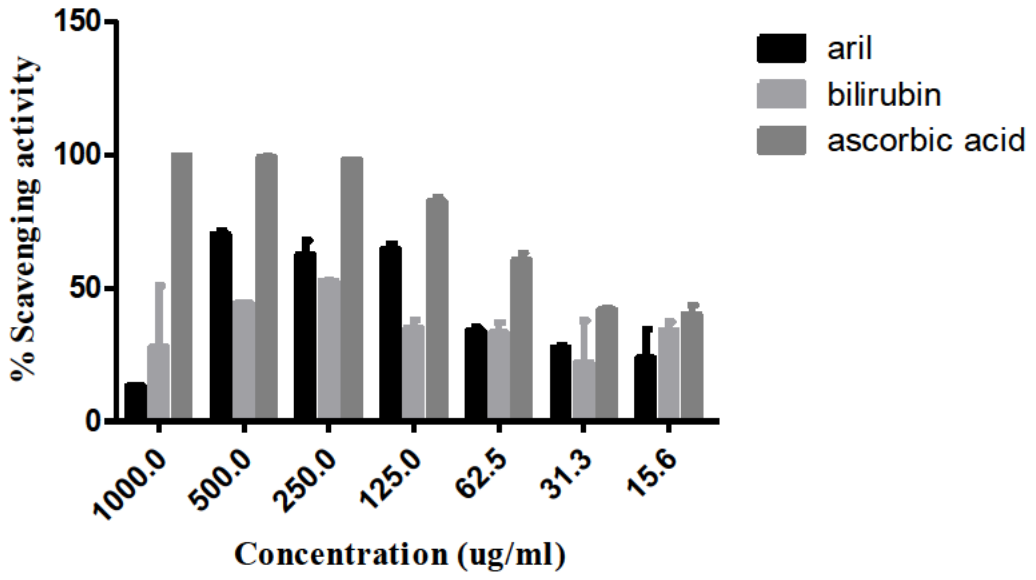


Figure 3: Antioxidant activity of the aril extract of *S. nicolai*, bilirubin and positive control ascorbic acid.

XTT assay

The aim of the cell viability assay was to determine which of the aril extract concentrations and bilirubin standard produced the most toxic effect on the HeLa cell line. The effect was observed for

24, 48 and 72 hours. The optimal dose and period were used in imminent methodologies to confirm apoptosis.

The concentration and percentage cell viability, was analysed using Graphpad prism 5 via the One Way Anova test and Kruskal-wallis test. The results revealed that the only significant difference shown between the different concentration of the arils and bilirubin at the different time period was within the 24 hour period.

Apoptotic cell death was seen after 24 hours (Figure 4) in the aril extract (52%) and bilirubin standard (20%). This was observed in the aril extract at a concentration of 250 µg/ml and the bilirubin standard at 7.8 µg/ml. Therefore, these two concentrations were used to further confirm apoptosis.

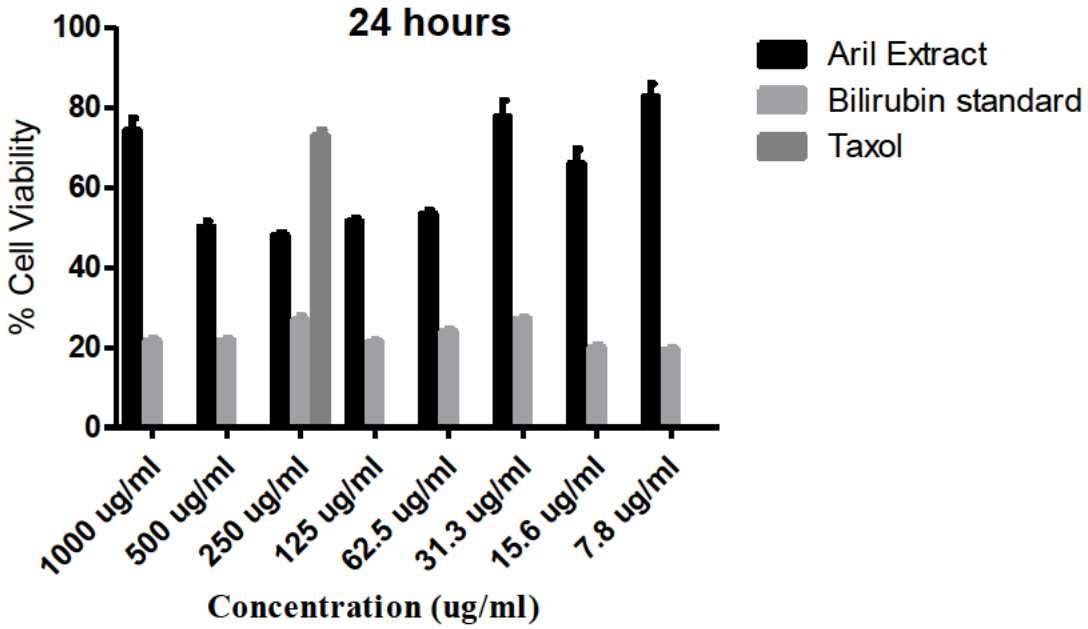


Figure 4: Effect of different concentration of the aril extract of *S. nicolai*, bilirubin and positive control (Taxol®) on HeLa cells after 24 hours.

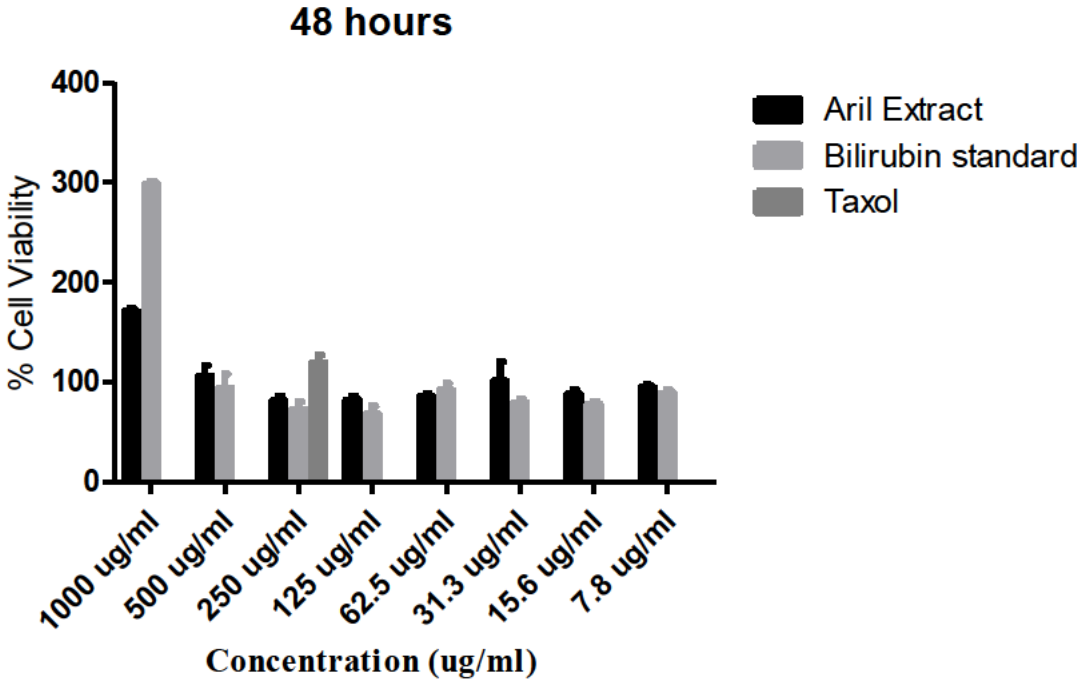


Figure 5: Effect of different concentration of the aril extract of *S. nicolai*, bilirubin and positive control (Taxol®) on HeLa cells after 48 hours.

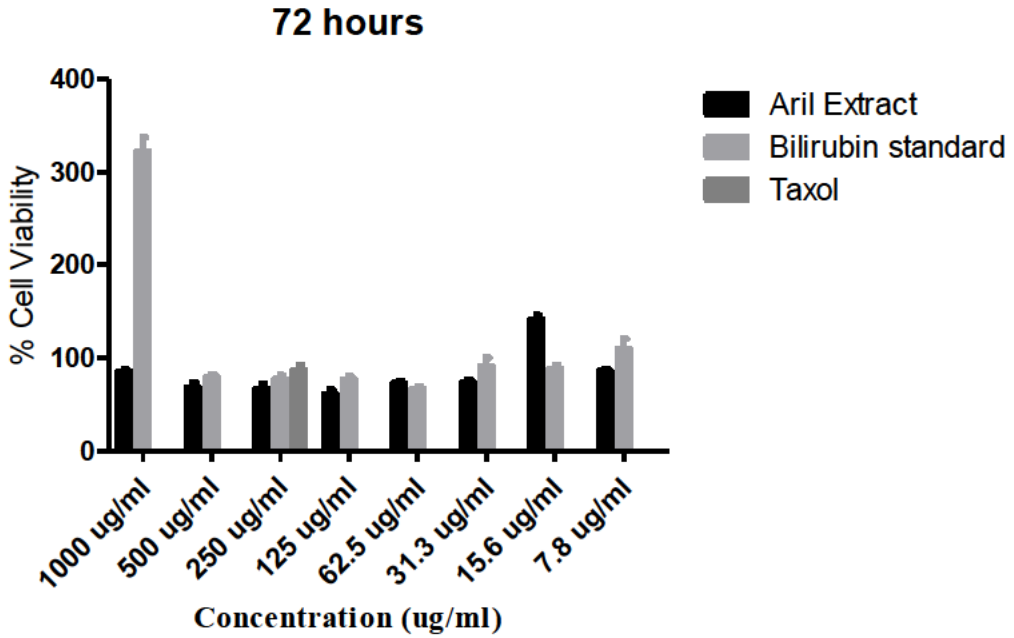


Figure 6: Effect of different concentration of the aril extract of *S. nicolai*, bilirubin and positive control (Taxol®) on HeLa cells after 72 hours.

Quantification of apoptosis

A difference in the number of viable cells in the untreated control and cells that were treated with the aril extract and bilirubin standard is evident. The aril extract and bilirubin standard produced more apoptotic cells than the positive control Taxol®. The aril extract facilitated twice the production of apoptotic bodies compared to the bilirubin standard on its own.

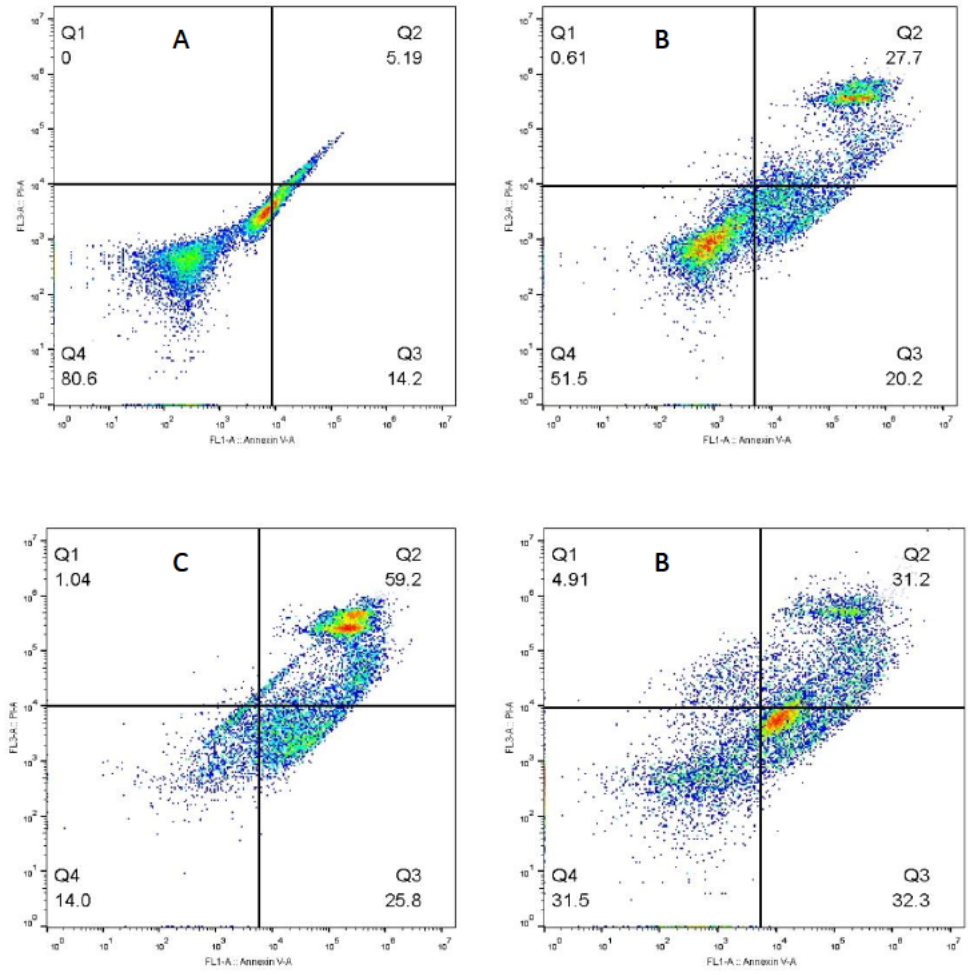


Figure 7: Flow cytometry analysis demonstrating the externalization of phosphatidylserine in HeLa cells treated for 24 hours with (A) untreated control cells (B) positive control Taxol® (250 µg/ml); (C) *S. nicolai* aril extract (250 µg/ml); and (D) bilirubin standard (7.8 µg/ml). Annexin V- PE Apoptosis detection assay results are presented in a scatter plot form, denoting the percentage of cells that are necrotic (upper left), viable (lower left), apoptotic (lower right) and late apoptotic (upper right).

Morphological study of apoptosis

Acridine orange and ethidium bromide double staining (Figure 8. A1) show viable normal cells with intact membranes. Figure 8B shows cells that were treated with Taxol®. There are only a few cells, as most of the dead cells were removed along with the medium. B1 shows some cells that were still viable and attached. B2 shows non-viable normal cells and B3 shows a viable apoptotic cell. Figure 8C exemplifies cells treated with the aril extract where C1 demonstrates viable apoptotic cells which show membrane blebbing, C2 is evidence of apoptosis as this shows nuclear material from surrounding cells and C3 demonstrates non-viable normal cells. Figure 8D is HeLa cells treated with bilirubin where D1 indicates non-viable normal cells, D2 shows normal viable attached cells and D3 illustrates viable apoptotic cells starting to blebb with an uneven membrane.

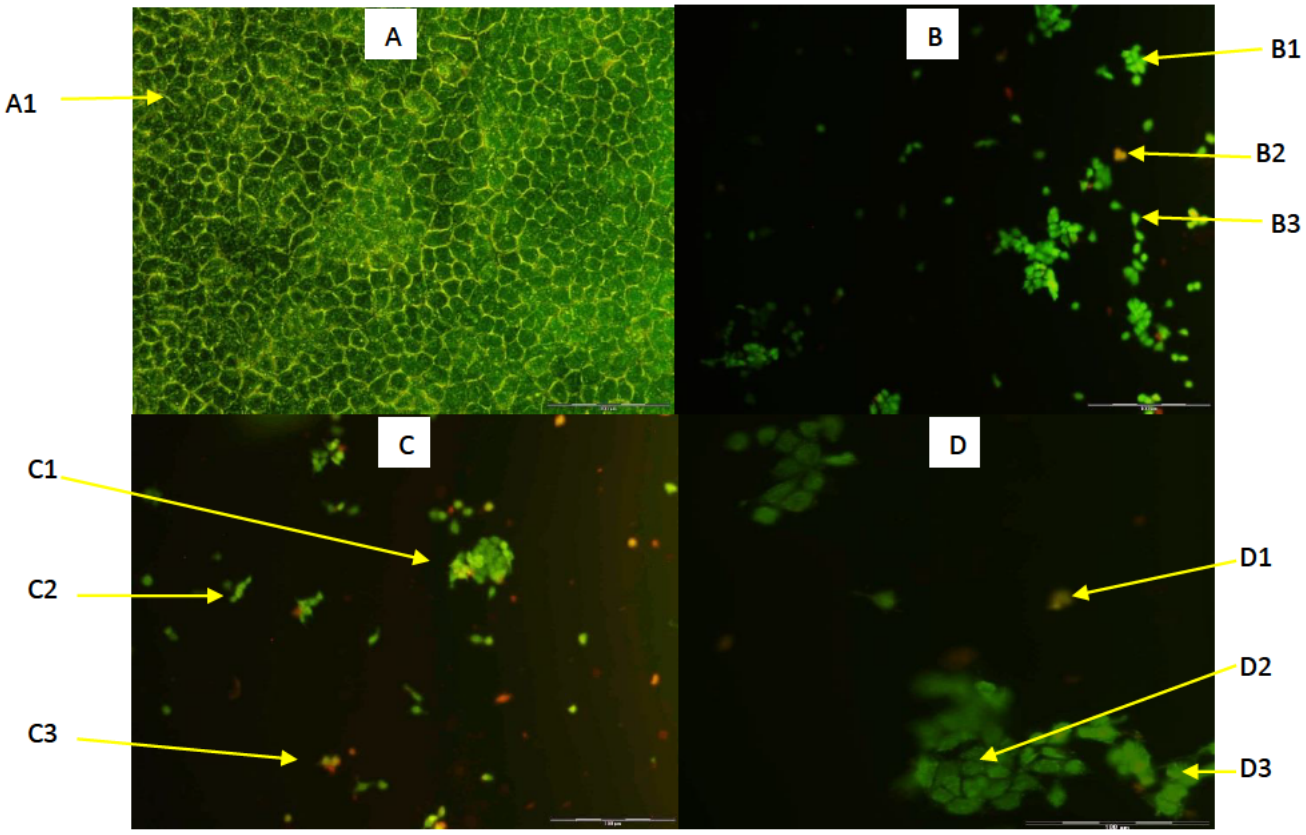


Figure 8: Fluorescence microscopic observation of cell membranes of HeLa cells treated with (A) untreated control cells (B) Taxol® (250 µg/ml); (C) *S. nicolai* aril extract (250 µg/ml); and (D) bilirubin standard (7.8 µg/ml).

Discussion

The disadvantage of conventional chemotherapy is the severe side effects. A successful anti-cancer drug should kill or debilitate cancer cells without causing extreme damage to normal cells (Sheehan and Hrapchak, 1987). Natural compounds isolated from medicinal plants, with potential anti-cancer properties, have been of increasing interest. Thus, the focus of this study was to screen the aril extracts of *S. nicolai* that contains bilirubin as well as to screen a bilirubin standard on its own, to determine which would act as a prominent inducer of apoptosis.

The antioxidant results of this study revealed that the aril extract from *S. nicolai* produced a higher radical scavenging activity than the bilirubin standard. This is an initial indication of the strength of the extract as an anti-cancer agent.

The XTT assay revealed the cytotoxicity of the HeLa cells that were treated with both the aril extracts and bilirubin showed a toxic effect after 24 hours, after which the cells disintegrated.

This study further investigated the type of cell death. The Annexin-V FITC detection kit was intended to detect specific changes in the cell surface membrane which are signature events of early apoptosis. The aril extract showed only a small number (14%) of cells that were viable. 25.8% underwent early apoptosis and 59.2% experienced late apoptosis. In cells that were treated with bilirubin, 31.5% were still viable, whereas 32.3% were going through early apoptosis and 31.2% late apoptosis.

Morphological features play a primary role in the description of cell death. Often, cell death includes both apoptosis and necrosis. The acridine orange–ethidium bromide stains further confirmed apoptosis with characteristic cell detachment and membrane blebbing. This double staining technique showed that the aril extract produced a larger number of detached smaller, more rounded cells than the positive control Taxol® and the bilirubin standard, thus showing effects of late apoptosis.

Conclusion

The results indicated that the seed arils of *S. nicolai* possess potential chemo preventive and therapeutic properties. The aril extract containing bilirubin showed enhanced activity compared to bilirubin on its own. These treatments indicate that the anti-cancer effects are related to two straight forward processes: inhibition of cell proliferation and initiation of apoptosis on HeLa cells *in vitro*. Whilst this study was an *in vitro* investigation, *in vivo* experiments can provide further insight into the use of this extract for the treatment of cancer.

References

1. American Association for Cancer Research (AACR) (2013). Novel serum biomarker bilirubin predicted lung cancer risk in smokers.
2. Diplock, A.T., Charleux, J.L., Crozier-Willi, G. 1998. Functional food science and defence against reactive oxygen species. *British Journal of Nutrition* 80 (Suppl 1):S77-S112.
3. Jiang, S., Lu, H., Liu, S., Zhao, Q He, Y., Debnath, A.K. (2004). N-substituted pyrrole derivatives as novel human immunodeficiency virus type 1 entry inhibitors that interfere with the gp41 six-helix bundle formation and block virus fusion, Antimicrob Agents Chemother, 48, 4349-4359.
4. Lathe, G.H. (1972). The degradation of haem by mammals and its excretion as conjugated bilirubin. Essays Biochem. 8, 107-148
5. Llesuy, S.F. and Tomaro, M.L (1994). Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. Biochimica et Biophysica Acta. 1223, 9-14.
6. McDonagh, A.F. (2001). Turning green to gold. Nat Struct Biol. 200, 98–200.
7. Monks, N.R., Bordignon, S.A.L., Ferraz, A., Machado, K.R., Faria, D.H and Lopes, R.M , et al. (2004). Anti-tumor screening of Brazilian plants. Pharmaceutical Biology 40, 603–616.
8. Murthy, N.J., Kotamballi, K.S., Guddadarangavvahally, P. (2002). Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extractusing in vivo models, Journal of agriculture and food chemistry, 50, 4791-4795.
9. Pandey, G. and Madhuri, S. (2006). Medicinal plants: better remedy for neoplasm. Indian Drugs 43, 869–874.
10. Pirone C. "Bilirubin: an Animal Pigment in the Zingiberales and Diverse Angiosperm Orders" (2010). FIU Electronic Theses and Dissertations. Paper 336. http://digitalcommons.fiu.edu/etd/336.
11. Sheehan, D.C., and Hrapchak B.B. (1987). Theory and practice of histotechnology, 2nd Edition; Battelle Memorial Institute, Columbus, OH.
12. Temme, H.M.E., Zhang, J., Schouten, G.E. and Kesteloot, H. (2001). Serum bilirubin and 10-year mortality risk in a Belgian population. Cancer Causes and Control. 12, 887-894.
13. Thomas, W., Sedlaka, M.S., Higginsonb, D.S., Paulb, B., Julurib, K.R. and Snydera, S.H. (2008). Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Pubmed. PMCID: PMC266404.

14. Zucker, S.D., Horn, P.S. and Serman, K.E. (2004). Serum bilirubin levels in the U.S. population: gender effect and inverse correlation with colorectal cancer. Hepatology. 40, 827–835.

Chapter 4

Journal article for publication 3:

Effect of *Strelitzia nicolai* seed arils containing bilirubin on Sprague Dawley rats

Depika Dwarka[1*], Sanil Singh[2], Veneesha Thaver[3], Mickey Naidu[4] and Himansu Baijnath[5*]

[1,5]School of Biological Sciences, University of KwaZulu-Natal, Westville Campus, Durban, South Africa. [2]Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa. [3]School of Laboratory Medicine and Medical sciences, Discipline of Physiology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa. [4]School of Health Sciences, Discipline of Pharmacology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa.

***Email:** depika.dwarka@gmail.com, baijnathh@ukzn.ac.za

Abstract

The discovery of the only human pigment bilirubin in the arils of *Strelitzia nicolai* Regel & Körn has opened up a new avenue in medical sciences. Previously, bilirubin was regarded as a toxic compound. However, recent case studies show that slightly elevated levels of bilirubin in the body may act as a powerful antioxidant. This study attempted to provide a basis for identifying potential toxicities related to arils containing bilirubin determined the acute toxicity of the aril extract of *S. nicolai*. Twelve Sprague Dawley rats were divided into four groups. Group A was the control and was administered 1% Tween 20. The other groups were orally dosed with the seed aril extract (250µg/ml/kgbwt) once a day. After 24 hours, 48 hours and 72 hours one experimental group and the control were sacrificed. Haemological and histological studies were thereafter conducted. The results obtained indicate a possibility of kidney damage. However, the possibility of the extract causing this

damage can be ruled out because this trend is seen in the control groups as well. This study reveals that an acute trial is insufficient in order to determine the toxicity of this extract. Further chronic studies need to be carried out.

Key words: *Strelitzia nicolai,* seed arils, bilirubin, aril extract

Introduction

S. nicolai is one of the seven species in the family *Strelitziaceae* and is included in the order Zingiberales. It is commonly known as the 'White Bird of Paradise plant'. *S. nicolai* is a herbaceous monocot native to South Africa, but is widely cultivated in warm temperate and tropical regions. *S. nicolai* can reach 8 meters in height and breeds in large clusters. The leaves are banana-like (hence the other common name 'bush banana') with long, stiff petioles and blades. The flowers are bluish and are shaped similar to a boat. It consists of three white sepals and 3 blue petals. The striking features of *Strelitziaceae* are the bird-like appearance of the inflorescence that is produced in layers. The fruit is a 3 lobed woody capsule containing black seeds (when fully matured) which has a bright orange aril (Van Wyk, 1993). *S. nicolai* is frequently used in landscaping, owing to its prominent features.

In 2009, a research team headed by Cary Pirone at Florida International University identified bilirubin in *Strelitzia reginae* and *S. nicolai*. Previously considered to be an "animal-only" pigment, bilirubin is best known as the yellowish colour associated with bruises and jaundiced patients. This became known as the only human compound that is found in a plant. This research team used high-performance liquid chromatography (HPLC) and HPLC/electrospray ionization–tandem mass spectrometry to identify bilirubin in the arils of *S. nicolai*. They found that in mature aril tissue bilirubin was present as granular bodies irregularly distributed throughout the cell (Pirone, 2010).

On average, nearly 250 to 400 mg of bilirubin are produced each day in humans. The unbound bilirubin is then free to cross the blood-brain barrier. Progressive accumulation of unconjugated bilirubin in the brain can result in neurological damage, kernicterus and eventually death (Brierley and Burchell, 1993). In humans, bilirubin is formed in cells by two sequential reactions, catalysed by haem oxygenase and biliverdin reductase. Bilirubin is generated when haem oxygenase catalyses the degradation of haemoglobin. This results in the configuration of biliverdin which is quickly converted into bilirubin by biliverdin reductase (Tenhunen et al., 1969). Further processing of bilirubin occurs in

hepatocytes where unconjugated (lipid-soluble) bilirubin is conjugated by uridine diphosphate–glucuronosyl transferase (UDP-GT) to a water-soluble form for excretion from the body (Hull and Agarwal, 2014).

Bilirubin is toxic when accumulated. It inhibits DNA synthesis and ATPase activity of brain mitochondria, and uncouples oxidative phosphorylation (Schiff et al., 1985). However recent case studies revealed that bilirubin may exercise some beneficial effects by virtue of its strong antioxidant properties (Breimer et al., 1995). Stocker et al., (1987) stated that bilirubin contains both antioxidant and immunomodulatory properties. There is also a report by Hopkins et al., (1996), suggesting that slightly higher serum bilirubin levels are coupled with a decreased risk for premature familial coronary artery disease. This recent revelation in medical sciences regarding bilirubin's formation and function, together with the discovery of this pigment in plants has led to the belief that there is an important function of bilirubin in the arils of *S. nicolai*. Therefore, this study was undertaken.

In a previous study, Dwarka et al., (*In press)* identified bilirubin's presence in the arils of *S. nicolai* using TLC and NMR. Subsequently, the antioxidant and anti-cancer activity of *S. nicolai* aril extract against a bilirubin standard were evaluated. The extract demonstrated good potential as an apoptotic inducer on Hela cells. Therefore, this study had to be conducted in order to evaluate the toxic effect of this extract.

Materials and methods

Collection of plant material

Fresh specimens of *S. nicolai* (Voucher specimen: Dwarka and Baijnath 1 Ward Herbarium UDW) arils were collected from Kwa-Zulu Natal, South Africa during 2016. The plants were identified using taxonomic keys.

Extraction of bilirubin from arils

Tissue (50 g) from the seed arils were ground using a mortar and pestle, extracted with 750 ml of chloroform for 72 hours and filtered through a Buchner funnel. The residue was re-extracted with 50 ml of chloroform. This procedure was repeated until the arils were colourless. The extract was subsequently air-dried to form a powdery residue which was re-dissolved in 1% Tween 20 and administered to the experimental rats.

Experimental Animals

Twelve Sprague Dawley rats, approximately 250g- 350g in weight, were supplied and housed in the Biomedical Resource Unit, University of KwaZulu Natal (ethics reference 075/14/Animal, Appendix 2). All animals were maintained at a temperature of 22˚C ± 1 ˚C, exposed to a 12-hour light-dark cycle and each group was accommodated in a 50 cm x 40 cm x 18 cm polycarbonate cage. All animals had ad libitum access to water and commercial rat chow (Meadow Feeds™ standard maintenance rat chow). General appearance and behaviour were monitored daily for clinical signs of pharmacological or toxic effects of the arils.

Treatments

Rats were divided into four groups of three. Group A (control group) was administered 1% Tween 20, daily. Groups B, C and D (experimental groups) were orally dosed with the aril extract (250µg/ml/kgbwt), daily. All rats were tagged and weighed at the commencement of the study. After 24 hours, group B and one rat from the control group were anaesthetised by Isofor (Safeline Pharmaceuticals) inhalation, and exsanguinated by cardiac puncture. Approximately 7 ml of blood was collected from each animal and sent to Global Labs (SANAS accredited). The parameters outlined in Table 1 were tested. Urine was collected and the dip stick method using Urinalysis test strip (Vadat), was used to extrapolate if potential abnormalities existed. Histological investigations of the liver, brain and pancreas were conducted. These organs were removed and fixed in cold, buffered neutral 10% formalin. Tissues were wax embedded and stained with haematoxylin and eosin. This procedure was repeated after 48 and 72 hours using groups C and D.

Statistical analysis

Data was analysed using graphPad Prism. The results of this study were expressed as mean ± standard error (Mean ± SE). To assess the significance of the differences between the control group and the three experimental rat groups, a statistical analysis was performed usinga two-way analysis of variance (ANOVA) for repeated measurements with the significance assessed at the 5% confidence level ($P<0.05$) and by a post hoc Bonferroni test. The significant difference was also noted between the different experimental groups (n=3).

Results

Group A compared to the control, produced P values > 0.05 for each of the tests except for alkaline phosphatase (ALP) which the P value <0.001***.Group B, compared to the control showed only a significant difference within the blood platelets (PLT)**. Group C showed no significant difference against the control group.

Group A compared to Group B showed significant differences with the PLT** and ALP** tests. Group A showed no significant difference in analysis to group C. Group B, compared to Group C, had no significant differences.

Table 1 below reveals that Group A rats had a lower red cell distribution width, mean platelet volume and higher potassium levels according to the standard range. Group B has a higher than normal potassium level. All four groups showed a lower than normal albumin level and higher direct bilirubin concentration.

Table 1: Blood analysis of Sprague Dawley rats dosed on aril extracs of *S. nicolai.*

Test	Reference range of rats	Control	24 hours	48 hours	72 hours
Full blood count					
White blood cell (WBC) (X 10^6/l)	4.0-12 2	5.7±0.4	5.03 ± 1.0	6.7±1.1	9.6±1.1
Red blood cell (RBC) (X 10^6/l)	5.4-12	9.2±0.1	8.7±0.3	8.8±0.1	8.3±0.2
Haemoglobin (HGB) (g/dL)	11.1-17.4	15.5±0.3	15.4±0.6	16.1±0.4	13.8±0.3
Haematocrit (HCT) (%)	36-52	46.7±0.3	51.6±5.2	49.0±1.0	46.5±0.9
Mean corpuscular volume (MCV) (%)	46-68	51.0±0.6	55.7±0.7	55.7±0.3	56.0±1.0
Mean Corpuscular Haemoglobin (MCH)	17.1-20.4	16.8±0.3	17.8±0.1	18.3±0.4	18.9±0.2
Mean corpuscular haemoglobin concentration (MCHC) (g/dL)	32.9-37.5	33.1±0.5	32.1±0.2	32.9±0.8	33.9±0.1
Red cell distribution width (RDW) (%)	11.1-15.2	12.5±0.6	10.8±0.2	11.3±0.8	10.9±0.2
Platelet (PLT) (10^3/µL)	638-1177	697.0±93.2	692.0±28.3	786.0±70.3	762.3±36.2
Mean platelet volume (MPV) (fl (μm^3)	6.2-9.4	6.5±0.2	6.0±0.1	6.4±0.1	6.3±0.1
Neutrophils (NE) (%)	6-50	8.2±1.9	8.3±1.7	6.1±1.8	5.5±1.4
Lymphocytes (LY) (%)	50-88	65.6±4.2	67.7±4.1	71.8±3.4	67.6±6.3
Monocyte (MO) (%)	10-40	24.4±2.5	23.5±3.6	21.3±2.3	26.3±4.9
Eosinophil (EO) (%)	0-6	0.3±0.05	0.1±0.03	0.4±0.03	0.1±0.05
Blood ammonia (BA) (%)	0-2	0.3±0.03	0.3±0.1	0.5±0.03	0.6±0.06
Liver function tests					
Total Protein (g/l)	59-84	64.3±1.2	64.7±0.7	63.7±0.3	63.7±2.4
Albumin (g/l)	32-46	19.3±0.3	17.7±0.9	17.0±1.1	17.7±0.9
Aspartate aminotransferase (AST) (IU/l)	54-262	179.0±8.2	164.3±18.9	146.7±8.1	115.3±5.8
Alanine aminotransferase (ALT) (IU/l)	52-144	76.7±11.1	59.0±8.0	68.3±8.4	64.3±6.4
Alkaline phosphatase (ALP) (IU/l)	40-250	218±84	163.3±48.4	253±31.8	217±45.8
Bilirubin Total (µmol/l)	4.0-8.2	8.0±2.0	8.0±1.0	4.0±0.0	8.3±0.9
Gamma-GT		3.3±3.3	3.3±3.3	2.0±0.6	1.0±0.6
Sodium (mmol/l)	129-150	141.3±0.9	145.3±0.7	145.3±0.9	143.0±0.6
Potassium (mmol/l)	4.3-6.3	7.9±1.0	8.3±0.1	5.8±0.1	9.3±0.1
Chloride (mmol/l)	100-109	102.0±1.2	104.3±0.9	103.0±0.6	103.0±1.6

Differences in statistical analysis of the different time intervals were prominent but there was no clinical significance because all of the parameters were within the normal rat range.

The urine analysis show relatively normal results when compared with the reference ranges of rats. However, traces of protein and nitrite were found in almost all the groups. In addition, leucocytes were also present in the experimental groups.

Table 2: Urine Analysis of Sprague Dawley rats dosed on aril extracs of *S. nicolai*

	Reference	1	2	3	4	5	6	7	8	9	10	11	12
Bilirubin	Negative	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Urobilinogen		Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm
Keytones	Negative	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Glucose		Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm	Norm
Protein	<30	Trace	30	30	30	30	30	30	30	30	Trace	Trace	30
Blood	Negative	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Nitrite	Negative	Neg	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Pos
Ph	5-7	6	6	6	6	6	6	6	6	6	6	6	6
Specific gravity	1.022-1.05	1.025	1.025	1.025	1.025	1.025	1.025	1.025	1.025	1.025	1.025	1.025	1.025
Leukocyte	Negetive	Ca.25	Ca.25	Ca 25	Neg	Ca.25	Ca 25	Ca.25	Ca.25	Neg	Neg	Neg	Neg

**Neg=Negetive, Norm=Normal, Pos= Positive*

Acute brain damage is characterized by neurodegenerative changes consisting of neuronal shrinkage, vacuolization of the cytoplasm, cell drop out, edema, microvacuolation of the neutrophil and hydronic cytoplasmic swelling of astrocytes. The granular layers of the treated animals did not have much difference compared with the control. If the dosed animals were affected, the granular layers would have been vacuolated. The hippocampus in all three treatments appear normal when compared with the control. The hippocampus in the dosed rats did not appear to be dense. On the whole, the brain cells look healthy with no evidence of the myelin rupture. The myelin usually starts to fracture about six hours after toxicity.

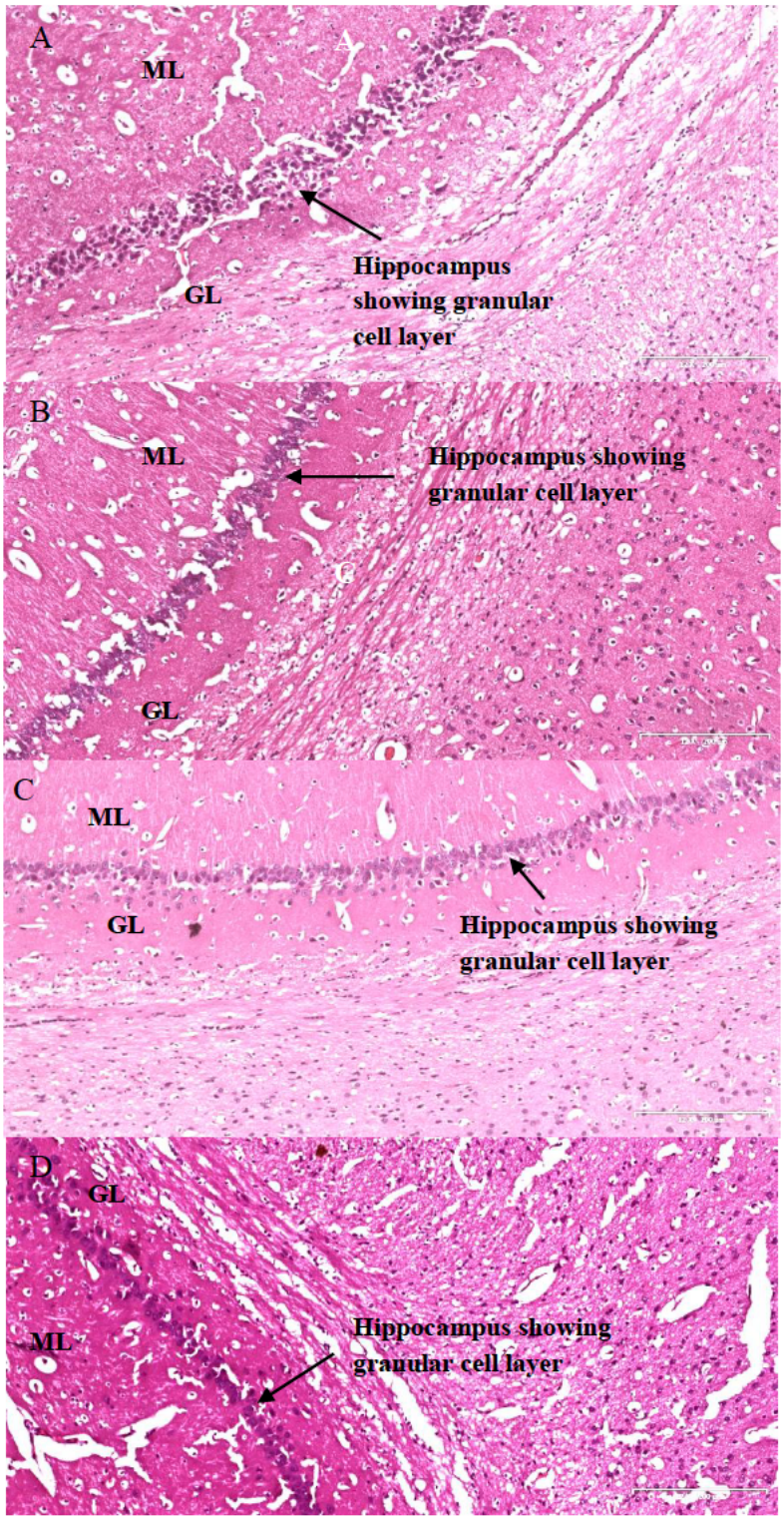


Figure 1: Photomicrograph of cross section of the cerebellar region of the rat brain, showing the molecular layer (ML) and granular layer (GL). Haematoxylin–Eosin staining of (a) control, (b) 24 hours, (c) 48 hours and (d) 72 hours after administration of *S. nicolai* aril extract containing bilirubin.

Acute toxicity of the liver tissue will manifest the following changes i.e. widespread cytoplasmic vacuolization, elaborate specks of necrosis, injured sinusoidal, intense fatty infiltration, swelling, ballooning, degradation, portal tract fibrosis with endothelial cell swelling, Kupffer cells around the central vein and the loss of cellular boundaries. The liver tissue of the treated cells appears normal when compared to the control. (Knight et al., 2001). Kupffer cells appear to be actively functioning with normal architecture. The hepatocytes appear to be normal with no irregular vacuolation and ballooning noted. The hepatocytes along the central vein appear normal with no fibrosis noted.

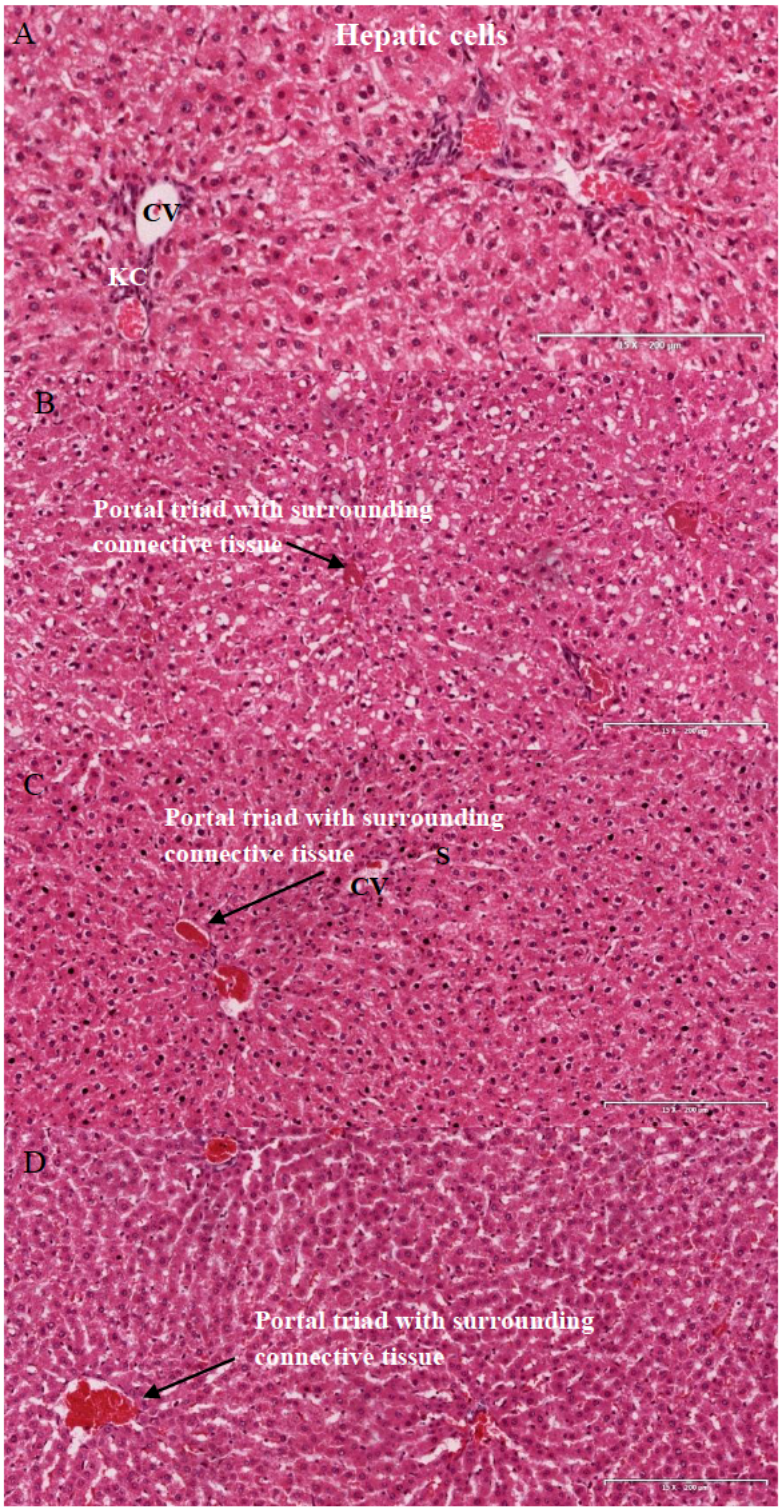


Figure 2: Photomicrograph of transverse section of the rat liver, showing normal hepatic cells, central vein (CV), and kupffer cells (KC) and sinusoids (S). Haematoxylin–Eosin staining of (a) untreated control, (b) 24 hours, (c) 48 hours and (d) 72 hours after administration of *S. nicolai* aril extract containing bilirubin.

Acute toxicity of the pancreas causes the improper secretion and activation of proenzymes within the pancreas. The activated pancreatic enzymes digest the cell membranes of the pancreas and set in motion an inflammatory response which intensify the vascular permeability of the pancreas. This leads to haemorrhage, edema, ischemia and necrosis of the pancreatic cells (Berardi and Montgomery, 2005). Figure 3 below shows normal configuration of the Islets of Langerhans in all three groups when compared with the control. The experimental groups do not show signs of expansion in the tissue septa and any signs of the disruption to the acinar arrangement.

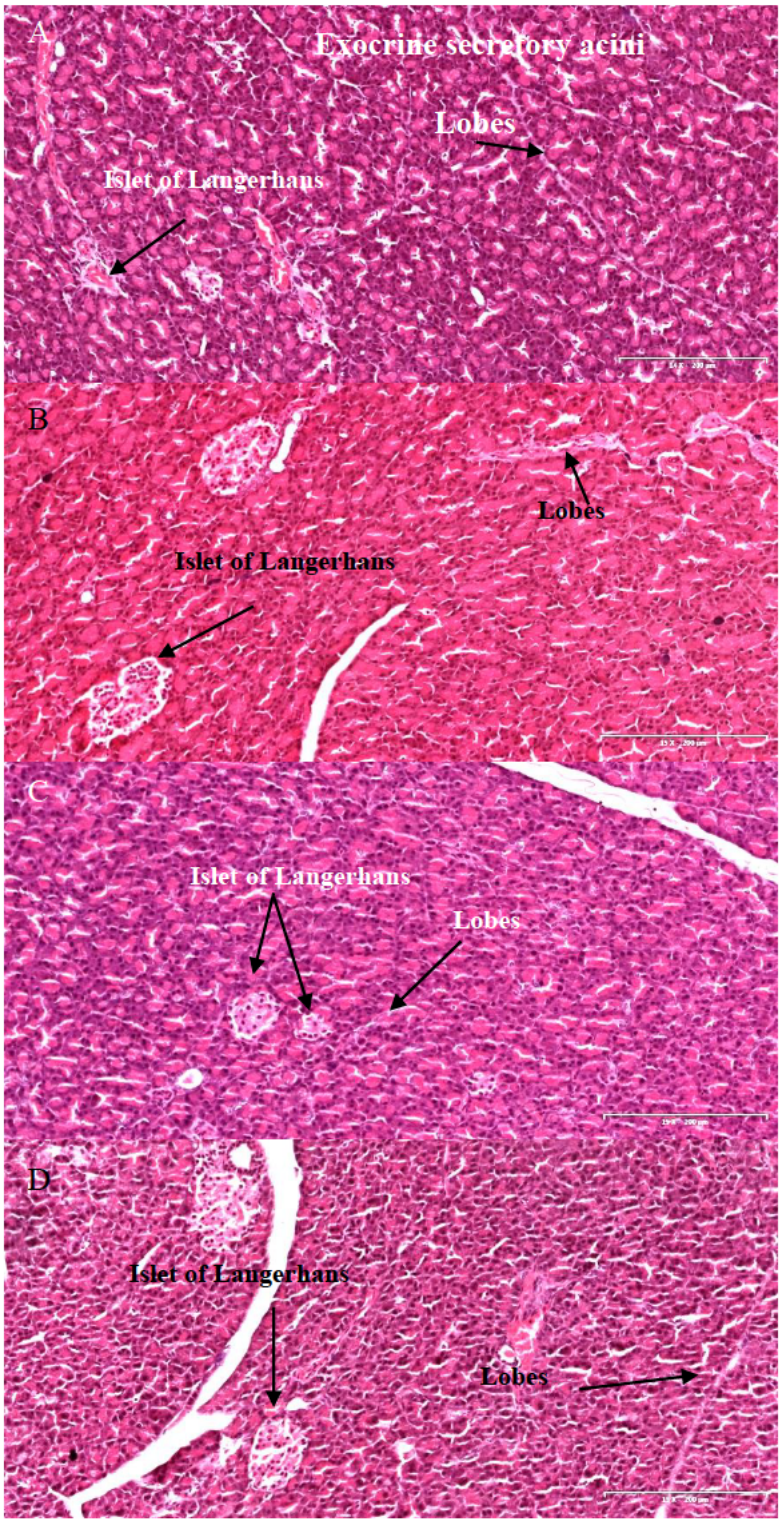


Figure 3: Photomicrograph of transverse section of the rat pancreas, showing exocrine secretory acini and the Islet of Langerhans. Hematoxylin–Eosin staining of (a) untreated control, (b) 24 hours, (c) 48 hours and (d) 72 hours after administration of *S. nicolai* aril extract containing bilirubin.

Discussion

Bilirubin-related toxicity can result in neuronal death or multisystem acute manifestations (Bhutani et al., 2004) enduring complications, including irreparable athetoid cerebral palsy, speech, vasomotor, auditory and other sensory-processing neuropathies. Even though for decades, bilirubin was assumed to be purely a excrement product of the haem catabolic pathway by greatest and a probable deadly composite at worst statistics from the last few years clearly show that slightly high serum bilirubin levels are vigorously related to have beneficial effects on the human body (Dwarka et al., 2017). This study examined the toxicity of bilirubin in the extract of *S. nicolai in vivo*.

Alkaline phosphatase (ALP) refers to a set of phosphomonoesterases which hydrolyse phosphate esters with optimal *in vitro* activity. The position of hepatic ALP on the sinusoid membrane suggest involvement in transport. Amplified ALP synthesis is seen during cholestasis with elevated bile acid concentration caused by intrahepatic or extrahepatic obstruction of the biliary tree (Vroon and Israili, 1990). There is a possibility that this extract, after 24 hours of administration, could have caused an obstruction in the biliary path or in the liver. This could possibly be due to the extract containing high lipid content and is, therefore, not broken down easily.

After 48 hours, the blood platelet count increased compared with the control but was still within the normal range. Drug induced cirrhosis can be excluded because this is associated with decreased level of platelets (Bureau et al., 2011) and not evidenced in the histology of the liver.

Potassium is critical for standard muscle development and function. It also assists sodium and calcium to sustain normal heart rhythm and control of the body's water equilibrium. However, if levels are too high it could be an indication of kidney damage. The potassium levels of all three groups and the control were significantly high. This could imply that the Tween 20 could have had an adverse effect on the kidney. Lister et al., (1987) showed that Tween 20 has been shown to decrease the bioavailability of a benzodiazepine receptor ligand 2 fold compared with the vehicle polyethylene glycol. Loscher et al., (1990) illustrated that the vehicle solutions can alter the bioavailability and increase or decrease the toxicity of experimental drugs and compounds.

Reduced blood albumin level is a condition called hypoalbuminemia and can occur when the body fails to absorb sufficient nutrients. This could imply that there is reduced kidney or liver function or there is an acute inflammation (Lyons et al., 2010). Patients with liver damage more or less indefinitely have hypoalbuminemia. This is due to the reduced synthesis by the hepatocytes and water and sodium retention that weakens the content of albumin in the extracellular space (Henricksen et al., 2001).

Proteinuria is the presence of increased quantities of protein in the urine (Beetham and Cattell, 1993). Elevated protein level in urine is an important indicator of progressive kidney damage (Iseki et al., 2003). Leucocytes in the urine give an indication that there is an inflammation in the urinary tract. The experimental groups showed signs of protein and leucocytes in the urine. This indicates inflammation in the kidney and urinary tract. Microorganisms that are accountable for the onset of urinary tract infection (UTI) create an enzyme that converts nitrates to nitrites. Nitrites in urine prove that a UTI exists. All of the control group and most of the experimental group indicate the presence of nitrites in the urine.

Cells in the different organs respond to toxicity in different ways. Sometimes, the cellular activity is increased or decreased with a distinguishable change in morphology and function of the mature cell type. In the treated experimental groups, the brain, liver and pancreas showed no signs of altered morphology (additional images found in Appendix 2). No imminent necrosis of cells was noted in any of the tissues that could have been attributed to the extract.

Conclusion

This study focused on determining the toxicity of *S. nicolai* aril extract containing bilirubin in an animal model. The results obtained in this acute study are inadequate enough to deduce if *S. nicolai* aril extract containing bilirubin is detrimental or harmless. The results do indicate signs of kidney damage and infection. This inclination is also found in the control samples. This indicates that the animals had this outcome before this study was commenced or it could also be that the Tween 20 caused adverse effects. A more extended study with more parameters needs to be carried out in order to verify the safety profile of this extract and its constituents.

Acknowledgments

This research was supported by the National Research Foundation (NRF) of South Africa. We would also like to thank Mrs Sohana Singh from the Department of Physiology UKZN and Dr Linda Bester from the Biomedical Resource Unit UKZN for their technical support and constant advice throughout this study.

References

1. Beetham, R. and Cattell, W. R (1993). Proteinuria: pathophysiology, significance and recommendations for measurement in clinical practice. Ann Clin Biochem 30:425-434.

2. Berardi RR, Montgomery PA. 2005. Pharmacotherapy: a pathophysiologic approach. New York: McGraw-Hill pp. 721–736.

3. Bhutani, V.K., Johnson, L.H. and Shapiro, S.M. (2004). Kernicterus in sick and preterm infants (1999-2002): A need for an effective preventive approach. Semin Perinatol. 2004;28:319–25.
4. Breimer LH, Wannamethee G, Ebrahim S *et al.* (1995) Serum bilirubin and risk of ischemic heart disease in middle-aged British men. *Clin Chem* 41, 1504–1508.
5. Brierley CH, Burchell B (1993). Human UDP-glucuronosyl transferases: chemical defence, jaundice and gene therapy. Bioessays 15:749–754.
6. Bureau, C., Métivier, S., D'Amico, M., Péron, J.M., Otal. P., Pagan, J.C., Chabbert, V., Chagneau-Derrode, C., Procopet, .B, Rousseau, H., Bosch, J. and Vinel JP. (2011). Serum bilirubin and platelet count: a simple predictive model for survival in patients with refractory ascites treated by TIPS. J Hepatol. 54(5):901-7.

7. Dwarka, D., Thaver, V., Naidu, M. and Baijnath, H. (2017). New insights into the presence of bilirubin in a plant species *Strelitzia nicolai* (Strelitziaceae). *AJCAM* 14(2):253-262.

8. Dwarka, D., Thaver, V., Naidu, M.., Koorbanally N. and Baijnath, H. *In Press*. In vitro chemo-preventative activity of *Strelitzia nicolai* aril extract containing bilirubin. *African Journal of Traditional, Complementary and alternative medicine*.

9. Henricksen JH, Siemssen O, Krintel JJ, Malchow-Møller A, Bendtsen F, Ring-Larsen. (2001). Dynamics of albumin in plasma and acidic fluid in patients with cirrhosis. *J Hepatol* 34: 53-60.

10. Hopkins, P. N., Wu, L. L., Hunt, S. C., James, B. C., Vincent, G. M., and Williams, R. R. (1996). Arterioscler. Thromb. Vasc. Biol. 16, 250–258
11. Hull, T. D. and Agarwal, A. (2014). The mononuclear phagocyte system in homeostasis and disease: a role for heme oxygenase-1. *Antioxid Redox Signal* 20:1770–1788
12. Iseki, K., Ikemiya, Y., Iseki, C. and Takishita, S. (2003). Proteinuria and the risk of developing end-stage renal disease. Kidney Int 63:1468-1474.

13. Knight, T.R., Kurtz, A., Bajt, M.L., Hinson, J.A. and Jaeschke, H. (2001). Vascular and hepatocelular peroxynitrite formation during acetaminophen toxicity: role of mitochondrial oxidant stress. *Toxicol. Sci*., 62: 212-220.

14. Limdi, J. K. and Hyde, G. M. (2003). Evaluation of abnormal liver function tests. *Postgrad Med J*. 79:307-312.
15. Lister, R. G., LeDuc, B. W., Greenblatt, D. J. and File, S. E. (1987). Poor bioavailability of CGS 8216 in a water/Tween vehicle following intraperitoneal injection. Psychopharmacology 91:260- 261.
16. Loscher, W., Nolting, B. and Fassbender, C. P. (1990). The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsan the influence of administration vehicles. EDileDsv Res. 7:173-18.
17. Lyons, O., Whelan, B., Bennett, K., O'Riordan, D., and Silke, B. (2010). Serum albumin as an outcome predictor in hospital emergency medical admissions. Eur J Int Med 21: 17–20.
18. Pirone C. (2010). "Bilirubin: an Animal Pigment in the Zingiberales and Diverse Angiosperm Orders". FIU Electronic Theses and Dissertations. Paper 336. http://digitalcommons.fiu.edu/etd/336.
19. Schiff, D, Chan G, Poznasky MJ (1985) Bilirubin toxicity in neural cell lines N115 and NBR10A. *Pediatr Res* 19 (9), 908–911.
20. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N., and Ames, B. N. (1987) Science 235, 1043–1048.
21. Tenhunen, R, Marver, H. S, Schmid, R. (1969). Microsomal heme oxygenase: characterization of the enzyme. *J Biol Chem*; 244:6388–6394.
22. Van Wyk. (1993). A photographic guide to trees of Southern Africa., Briza Publications, Pretoria.
23. Vroon, D. H. and Israili, Z. (1990). Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition. Boston: Butterworths.

Chapter 5

Journal article for publication 4:

Preparation techniques of *Strelitzia nicolai* aril tissue for scanning electron microscopy

Depika Dwarka[1*], Nelisha Murugan[2], Veneesha Thaver[3], Mickey Naidu[4] and Himansu Baijnath[4*]

[1,4]School of Biological Sciences, University of KwaZulu-Natal, Durban, South Africa. [2]Microscopy and Microanalysis Unit, University of KwaZulu-Natal, Durban, South Africa. [3]School of Laboratory Medicine and Medical sciences, Discipline of Physiology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa. [4]School of Health Sciences, Discipline of Pharmacology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa.

Email: depika.dwarka@gmail.com

Key words: Sample preparation, chemical fixation, partial vacuum SEM, cryo-SEM, freeze drying.

Research highlights: The main aim of this study was to uncover the most accurate preparation procedure for seed arils in order to be viewed using the SEM. After evaluating six different processes, cryo-fixation of fresh material was ultimately the best option that showed the best preservation of aril tissues.

Abstract

Objective: Scanning electron microscopy (SEM) is commonly used in the analysis of seeds and seed arils. However, so far a specific methodology to prepare the seed arils of *Strelitzia nicolai* Regel & Körn. has not been evaluated. This study assessed six different sample preparation methods for the imaging of seed arils of *S. nicolai. Materials and methods:* The methods include a) fresh, and uncoated samples viewed under a partial vacuum SEM (VPSEM) b) cryo-fixation of fresh material followed by freeze drying for 48 hours, c) cryo-fixation of fresh material followed by freeze drying for 24 hours, d) chemical fixation using a solution of formalin: acetic acid: alcohol (FAA), e) conventional chemical fixation of material using glutaraldehyde (GA) and f) cryo-fixation of fresh material, followed by sputter coating and viewing under a high vacuum on a cold stage. Preparation methods were evaluated based on the quality and integrity of the resultant sample as observed in the images taken. *Results:* The arils that had undergone preparation methods a - e appear to have collapsed and/or shrunk. SEM sample preparation via conventional chemical fixation techniques lead to distortion of the sample. Specific preparation procedures may need to be tested for a specific plant part needed to be examined, as was determined in this study. *Conclusion:* From this investigation, it can be concluded that imaging seed arils of *S. nicolai* using a cryo-SEM showed good preservation of the sample and the least distortion of the tissue.

Introduction

The invention of the scanning electron microscope (SEM) in the 1940s embodied a major breakthrough in the study of microstructure and composition of material. Since the SEM became commercially available, it has become an extremely valuable characterisation tool for scientists. The SEM combines high resolution imaging with a large depth of field rendering 3D like images. These images are largely created by short wavelengths of electrons and their ability to be focused using electrostatic and electromagnetic lenses (Stokes, 2008)

The SEM functions at high vacuum to eliminate gas molecules interacting with the primary electron. For metallic material, SEM sample preparation is straightforward. However; numerous methods are considered when dealing with biological specimens that are made up of predominantly water and oil. Usually, this involves chemical fixation and drying to remove volatile substances (Stokes, 2008).

Sample preparation is crucial, the critical concern being that the specimen prepared be an accurate representation of the natural sample (Boyde and Boyde, 1980).This implies that all biological material being prepared for SEM first needs to be fixed. Ideally, fixation is to preserve the structure of living tissue with no alteration from the natural state. It should also protect the tissue against disruption during subsequent exposure to the electron beam (Bozzola and Russel, 1992). Frequently, delicate plant material which is prepared by conventional SEM techniques incorporates chemical fixation, ethanol dehydration critical-point drying and sputter coating. Normally a chemical fixative, such as glutaraldehyde is used. Glutaraldehyde is a five carbon compound containing terminal aldehyde groups. It fixes biological material by cross linking proteins. The aldehyde groups react with α-amino groups of lysine in adjacent proteins, thereby cross linking them. Another chemical fixative that is not usually used in SEM is coagulative Formalin-Acetic-Alcohol (FAA). FAA works by forming bonds with the amino and peptide groups of proteins. Although FAA is a damaging fixative, it penetrates rapidly. (Hayman and Philip, 1970). Therefore, plant material can be stored indefinitely in FAA (Berlyn and Miksche, 1976).

Dehydration is a process that follows fixation. Dehydration allows for the water in the tissue to be replaced gradually by using a graded series of dehydrating agents. Ethanol and acetone are the most general agents used. Subsequent to dehydration, the dehydrant is displaced with pressurized liquid carbon dioxide in a critical poingt dryer (CPD). Following application of several changes of fluid, heat is then applied. This causes the pressure in the vessel to rise. Ultimately, the fluid will reach critical point; i.e. particular temperature/pressure that the fluid transits from a liquid to a vapour phase (Araujo et al., 2003). Before viewing and image analysis, the sample is sputter coated. Argon ionises gold in a vacuum and deposits it uniformly on the material. This ensures that it is conductive under the electron beam. Conventional chemical fixation may cause the specimen to shrink and dehydrate extensively to a degree that it looks different to the native sample (Parsons et al., 1974). This is a problem when working with biological material which is basically made up of 98% water (Pathan et al., 2009). Therefore, caution needs to be taken in the preparation steps in order to ensure that the inherent structure of the organism is retained. Researchers Hardy et al., (1995) also failed to achieve acceptable surface preservation for *Dactylis glomerata* and *Elymus canadensis* leaf samples using CPD after fixation with conventional glutaraldehyde. Pathan et al., (2009) fixed bean leaf samples in 2.5% glutaraldehyde and 2% osmium tetroxide, followed by dehydration in a graded series of ethanol and finally CPD.

They achieved acceptable preservation. However, epidermal cells appeared to be shrunken, especially around stomata of bean and *Chenopodium*. They concluded that this shrinkage of tissue probably occurred during dehydration rather than drying (Boyde and Maconnachie, 1983).

One of the more recent methods that allow biological specimens to be imaged without preparation is Variable Pressure Scanning Electron Microscopy (VP-SEM). This instrument has become an important tool for the imaging of hydrated specimens with low conductivity. In the VPSEM, gas is used to enhance signal detection (Futing, 2003). The formation of positive ions by certain products produce an insulation for specimens, thereby allowing the specimen to be visualized without the need for a conductive coating (Stokes, 2008). This allows for the specimen to be viewed in its natural state. Callow et al., (2003) observed that the mechanism of adhesion of spores of the marine alga *Enteromorpha* was through a gel-like adhesive pad which was visible in ESEM rather than the fibrillar structure seen in SEM, as previously thought.

Some biological samples cannot with stand conventional chemical preparation or pre-treatment with varying pressure as they collapse and distort (Boyde and Maconnachie, 1979). Conventional SEM preparation regimes often cause up to 20-30% of shrinkage of biological tissues. The freeze drying method is used whereby samples are plunged in slush nitrogen (−210 °C) and then subsequently transferred to a chamber that allows the solid to gradually undergo sublimation to a gaseous phase. (Pathan et al., 2009). Liquid nitrogen slush is used to allow for rapid freezing rates. This avoids ice crystal formation. This is another technique used to preserve biological specimens containing water. This improved method allows less shrinkage of the specimen than critical point drying (Takao and Hitoshi, 1988). An additional method stemming from the freeze drying is cyro-SEM. This method is indispensable for the examination of wet or beam sensitive specimens. It eliminates the need for conventional preparation techniques such as critical point drying or freeze-drying, and permits observation of the specimen in its 'hydrated state'. Tsou et al., (2015) used cryo-SEM to examine the anther development of Poaceae for the first time. They showed that the peripheral arrangement of pollen grains is a pre-requisite for pollen maturation.

Strelitzia nicolai, an indigenous plant of South Africa, normally known as the 'White Bird of Paradise' or 'Wild Banana' is widely used in all parts of the world as a landscaping showpiece. Recently the focus of this plant has shifted to medical sciences. *S. nicolai* is among the few plants which have been confirmed to contain bilirubin, which is the only animal pigment found in a plant

(Pirone, 2010). This pigment was dominant in the seed arils of this species. An aril is a specialized appendage from the funiculus and covers the seed. In *S. nicolai*, the arils are dense bright orange tufts. Boesewinkel and Bouman (1984), define the aril as a 'fleshy seed appendage'. These delicate appendages (seed arils) if not pretreated suitably can alter aril quality considerably by introducing artefacts. Therefore, the objective of this study was to compare the appearance of the aril tissue prepared with different SEM methods. Conventional preparation techniques for scanning electron microscopy are widely used for biological material (Talbot and White, 2013). However, plants vary in their tissue characteristic which makes them relatively difficult to preserve in their fundamental form as compared to animal or insect tissues. Specific preparation techniques need to be tested for the specific plant part needed to be examined, as was determined in this study.

Materials and methods

S. nicolai seeds (Voucher specimen: Dwarka and Baijnath 1 Ward Herbarium UDW) were collected at different stages of development from plants at the University of KwaZulu-Natal, South Africa. Arils were removed very carefully and subjected to pre-treatment.

Stereomicroscopical analysis

Fresh seeds with attached arils were collected and viewed using the Nikon AZ 100 microscope attached to a high performance camera. Arils attached to the seeds were photographed using 1X objective, the arils only were photographed using 4X magnification and Z-stacking was performed on these arils in order to create a single sharp image as well as to increase the depth of focus using the NISD Elements D 3.0 software.

Light microscope imaging

Fresh seeds were collected, aril strands separated and the individual strands were permanently mounted on slides and viewed using the Nikon Eclipse 80i light microscope. The arils were imaged at 4X magnification.

Variable pressure SEM (VP SEM)

Arils of *S. nicolai* were collected and immediately viewed using the Zeiss EVO LS15 VP SEM. This mode of microscopy allows biological samples to be examined without going through a number of preparation stages. This is achieved because the VP SEM allows hydrated or partially hydrated material to be viewed without coating with heavy metals. Samples can be viewed in this state owing to the presence of water vapour in the chamber which aids in conductivity. It also uses a modified detector to detect the secondary electrons, therefore allowing hydrated samples to be viewed in its natural state.

Freeze drying

Fresh aril material was collected a few minutes before commencing this method. The metal sample holder of the freeze-dryer was removed from the instrument and pre-cooled using liquid N2 (approximately 15 minutes). A liquid nitrogen slush was produced by placing liquid nitrogen in a vacuum. At atmospheric pressure, liquid nitrogen is -196°C and when in a vacuum and the pressure lowered, it goes from -196°C to -210°C which is now liquid nitrogen slush. A liquid nitrogen slush is used instead of liquid nitrogen because liquid nitrogen boils when it comes into contact with a warmer object (the sample), creating nitrogen gas which envelopes the sample and impedes the sample freezing rate. The slush is a mixture of solid and liquid nitrogen, hence faster cooling. This preserves the sample in its natural state. The aril tissue was plunged into the liquid nitrogen slush and placed onto a pre-cooled sample holder. The sample holder was moved into the freeze-drying apparatus and the pre-cooled chamber was then evacuated of air to form a vacuum. The continual vacuum pumping permitted the water to sublimate. Aril tissue was allowed to freeze dry (Edwards freeze dryer) for 24 hours and a separate batch for 48 hours. Thereafter, the dried specimens were mounted on stubs with carbon tape, coated with gold in a sputter coater and viewed with the Leo 1450 SEM.

Chemical fixation using FAA

Arils were fixed for 24 hours in FAA. Samples were then rinsed in 0.1phosphate buffer, transferred in a graded ethanol series (10%-100%) and then dried in a critical point drier (Quorum K 850). After which, the samples were mounted on stubs, coated with gold (200-220 A in thickness) using a Quorum K150 RES Sputter Coater. The specimens were viewed with a Zeiss LEO 1450 SEM. at an acceleration voltage of 5 kV.

Chemical fixation using glutaraldehyde

Arils were fixed for a minimum of 24 hours in 2.5% glutaraldehyde in 0.1Mphosphate buffer (pH 7.2) and post-fixed in 2% osmium tetroxide for 1 hour at room temperature. The samples were then rinsed in phosphate buffer, transferred to a graded ethanol series (10%-100%) and then dried in a critical point drier (Quorum K 850). Following this, the samples were mounted on stubs, coated with gold (200-220 A in thickness) using a Quorum K150 RES Sputter Coater and were viewed with a LEO 1450 SEM. at an acceleration voltage of 5 kV.

Cryo-SEM

Fresh aril tissue was mounted on a specimen block using a colloidal graphite paste. Once the aril tissue was in position, it was plunged into nitrogen slush in a Quorum PP3000T cryo-SEM preparation system. Once frozen, the whole unit was transferred into the cryo-preparation chamber at a temperature of 90°C and a pressure 3 Pa (high vacuum conditions). After approximately 5 min (sublimation stage), the sample was sputter coated with 8 nm platinum for 3-4 minutes and thereafter transferred under vacuum onto the cooled stage of the Zeiss Ultra Plus FEGSEM.

Results

To identify a robust preparation methodology that allows aril tissue to be viewed with the least amount of alteration in tissue surface and morphology, the effects of six different protocols on the preservation of aril tissue of *S. nicolai* were compared. Specific methodology effects were assessed by imaging the aril morphology and dimensions as compared with the original sample. Figure 1 shows the five different stages of aril development in *S. nicolai* seeds. The colour of the aril changes from opaque to yellowish and finally to a deep orange. For the purpose of this study, only the findings based on the early stage of aril development was reported. This is owing to the fact that the early stage was the most delicate and difficult to prepare for SEM. Figure 2A shows stereo micrographs of stage 1 aril (4X) and 2B shows an individual aril photographed using the light microscope at 40X.

Figure 1: Stereo micrographs of the 5 different stages of aril development in seeds of *S. nicolai* as categorised in this study, showing (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5 arils attached to the seeds.

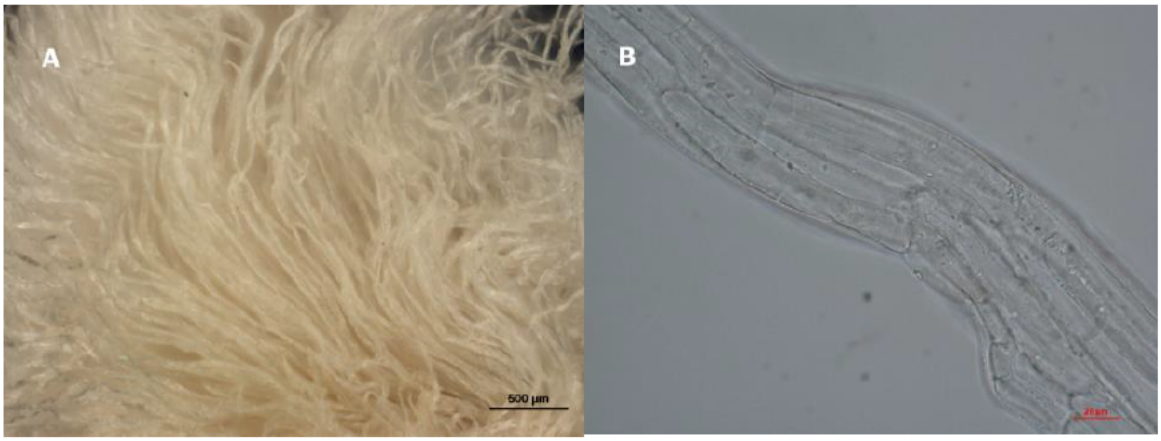


Figure 2: Photomicrograph showing stage 1 arils, at A captured using the stereomicroscope and B using the light microscope.

Figure 3 shows fresh unprepared arils that were viewed using the VP SEM. A visual comparison of *S. nicolai* aril tissue using this method shows reduction and shrinkage in aril tissues in a small number of regions. However, it was noted that the tissue had to be viewed rapidly because as time passed more areas of the aril endured drying out (indicated with arrows). Furthermore, as pressure increased in the chamber an increased number of areas dried out. The preparation time, as compared to the other methods, is quite swift conversely, the rate that the young aril tissues deteriorate is quite vast and therefore this method is not suitable for delicate tissue such as arils. This method is usually suitable for samples that can be viewed quite quickly after removal (additional images in Appendix 3).

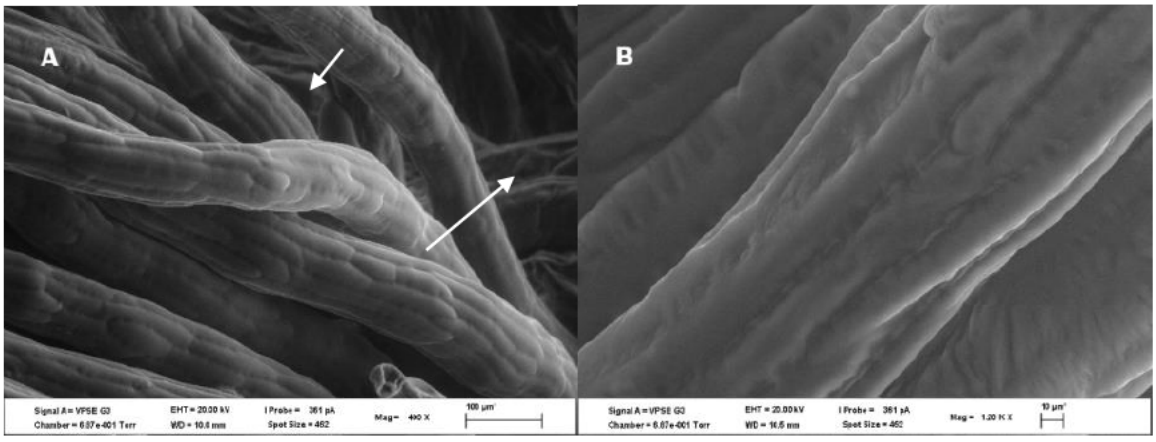


Figure 3: Fresh arils viewed with VP SEM at lower magnification (A) individual arils had collapsed. At higher magnification the aril surface was longitudinally folded.

Freeze dried aril tissues for 48 hours (Figure 4) show one of most damaging methods to aril tissues. This method resulted in partial collapse, folding and shrinkage of aril tissue. Tissue morphology and tissue dimensions were negatively affected using this method. Furthermore, the period that the aril

tissue was placed under vacuum within the SEM had an adverse effect on the shrinkage rate of the samples.

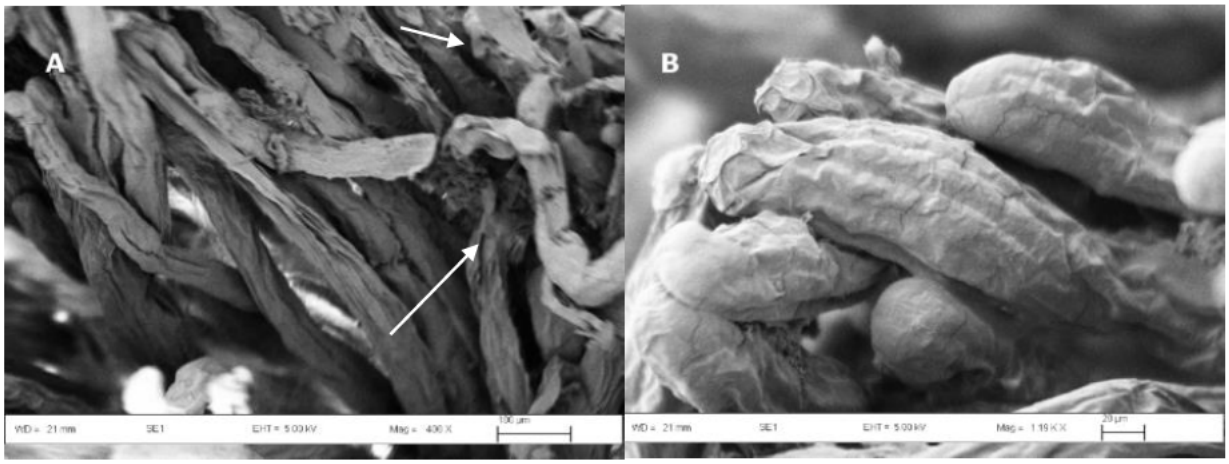


Figure 4: Fresh arils freeze dried for 48 hours at lower magnification (A) individual arils had collapsed and (B) at higher magnification.

Figure 5 shows freeze dried aril (24 hours). This also shows surface shrinkage (indicated with arrows). This was less apparent than samples that were freeze dried for longer periods (48 hours). Another drawback of this method is that freeze dried samples are very difficult to handle and they break very easily, hence the broken aril in Figure 5A. The freeze drying methods are relatively quiet time consuming as compared to some of the other methods used in this study.

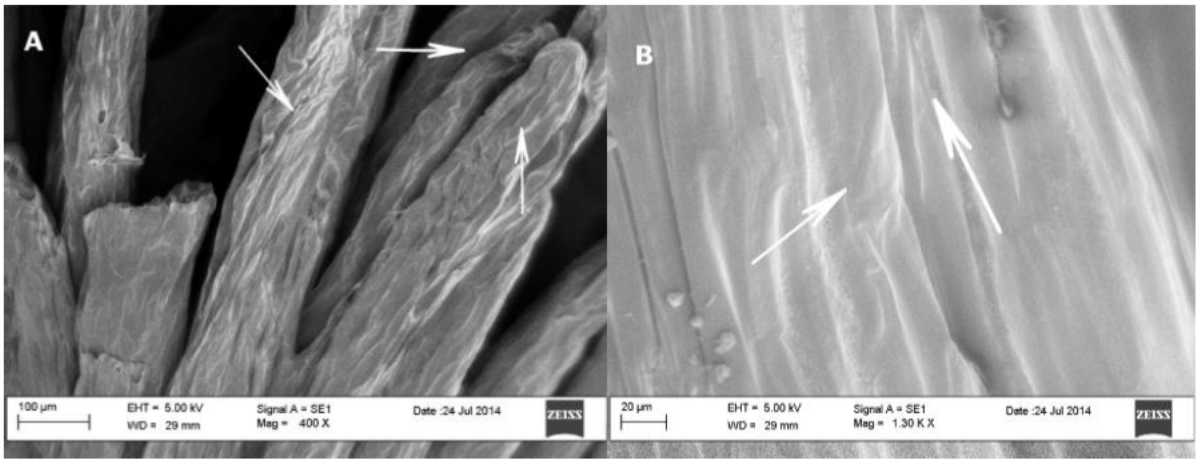


Figure 5: Fresh arils freeze dried for 24 hours at lower magnification (A) individual arils had collapsed and (B) at higher magnification.

Arils that were fixed in FAA and then dehydrated, critical point dried, gold coated and viewed. Figure 6 did not shrink or distort as much as freshly coated or freeze dried samples, the aril tissue was

difficult to view as it produced charges caused by non-conducting areas in the sample. The aril structures are like tiny hairs and coating deposition is from the top (above the sample). Hence, not all areas of the tissue could be coated evenly, thus resulting in non-conductive areas therefore the increased charging.

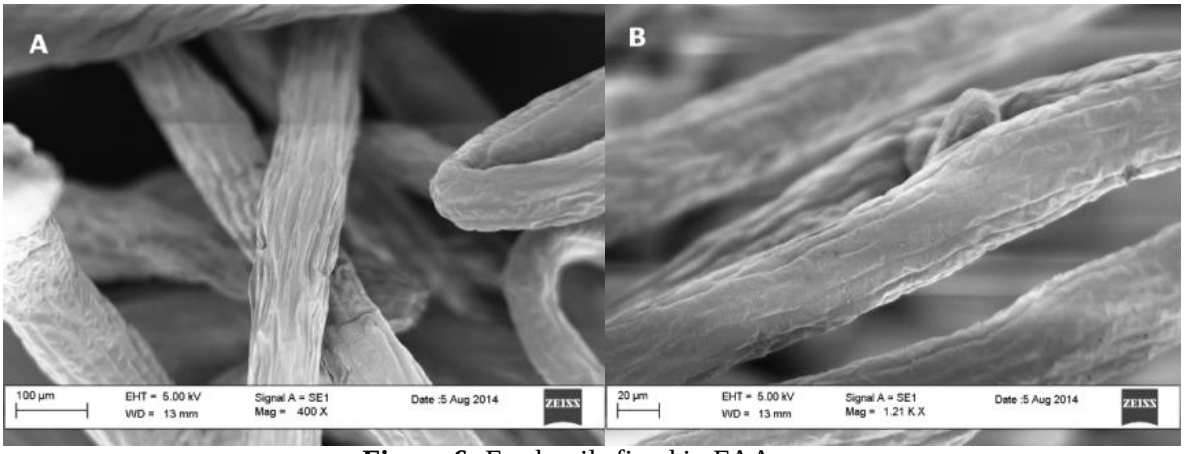


Figure 6: Fresh arils fixed in FAA

The arils fixed in glutaraldehyde and osmium tetroxide, serially dehydrated with ethanol, critically point dried and then sputter coated with gold shrunk and looked different to the arils from their natural state. This method (Figure 7) clearly distorted aril tissue morphology and was also time consuming. Its only advantage is that the samples are easy to handle, hence the sample had less mechanical damage.

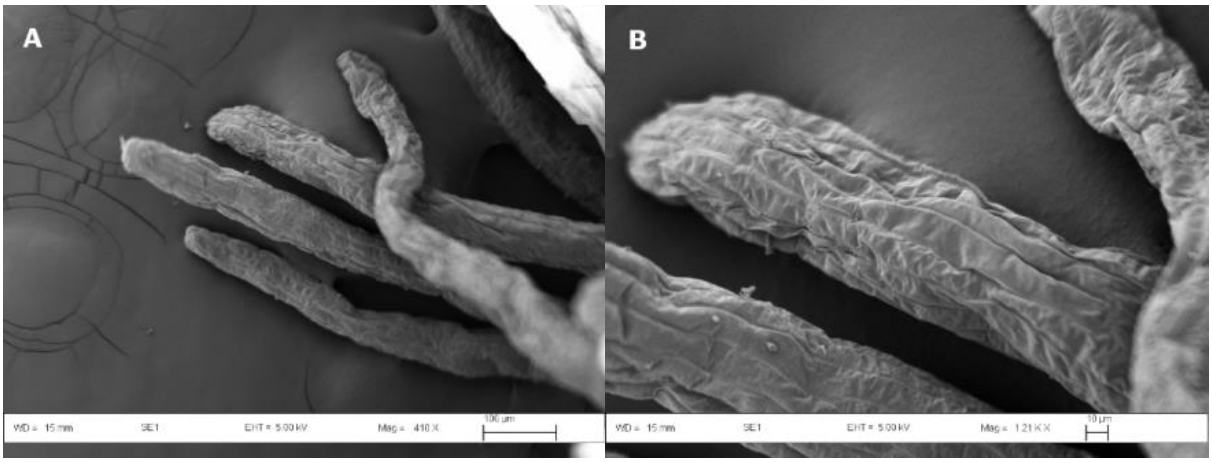


Figure 7: A (400X) and B (1.2 KX) shows arils prepared with conventional glutaraldehyde

The arils viewed with the cryoSEM did not appear dehydrated and their surface structures remained unaltered. Compared with all the other methods tested in this study, this method seems to be the most suited with the least distortion of the sample. It is also a very quick method.

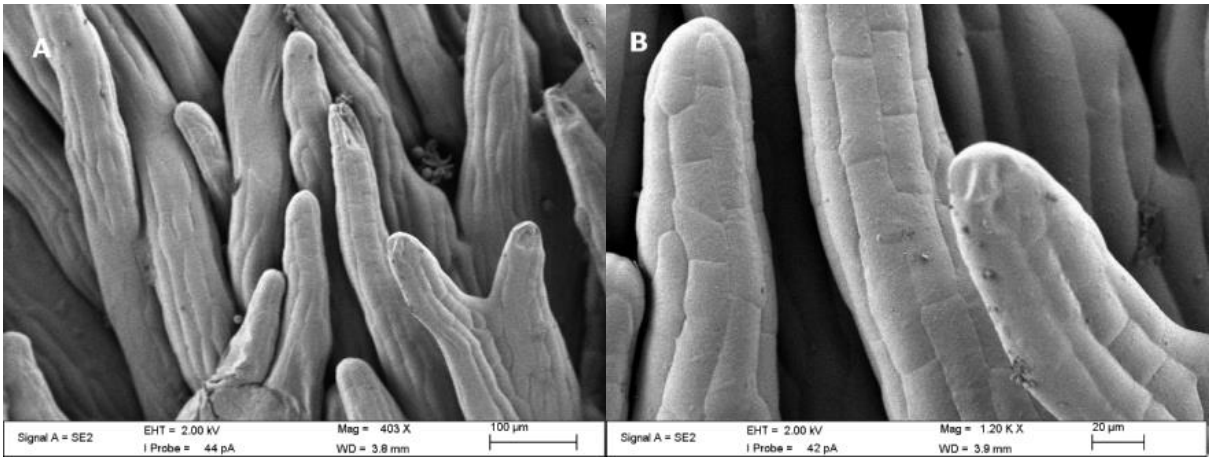


Figure 8: A (400X) and B (1.2 KX) reveals arils that were viewed with the cryo-SEM.

Discussion

In this study, six different methods to treat delicate seed arils from *S. nicolai* for SEM were examined. The six methods were assessed on the general appearance of the arils, and shrinkage or distortion of the tissue. The results obtained in this study fortify preceding observations that SEM processing methodologies vary for different types of tissues. Critical point drying following chemical fixation with glutaraldehyde and/or osmium tetroxide, is the most common preparation method used for biological samples. For seed arils of *S. nicolai,* this is not advocated. It was found that the fixatives caused deformation of aril tissue by negatively influencing the drying process. Possibly the fixative was at too higher concentration. Lapsley et al., (1992) reported that critical-point drying and fixation using conventional glutaldehyde caused apple tissue to become dehydrated which led to artefacts when viewed.

Fresh material viewed with the VP SEM did not produce good quality images because some arils were hydrated and looked in their natural state, whereas others were totally dehydrated and shrunk Pathan et al., (2010) reported 'true-to-life' low magnification images of *Chenopodium* leaves. However the sample collapsed during imaging at high magnification. This seems to be a good method to use for limited fresh samples that one can view immediately.

FAA is normally used in light microscopic sample preparation. The advantage of FAA as a fixative is that it has a higher rate of penetration than either glutaraldehyde or osmium tetroxide so that large blocks of tissue are well fixed. Fixation with FAA before carrying out the dehydration procedure produced a fair amount of conservation of aril tissue. However, arils preserved in FAA produced images that 'charged' a lot. Charging is caused by non-conducting samples that cause an accumulation of static electric charge on the specimen surface. This static charge influences the

electron signals and hence deteriorates the image information. The aril tissues protrude and do not lie flat on the surface of the stubs. This could be one of the reasons that even though the samples were gold coated twice poor images were recorded.

This study also shows that the amount of time delicate plant material spends in the freeze dryer also affects the shrinkage of the specimen. In this study we saw that the arils that were in the freeze drier for 24 hours were far less distorted than those that were in the freeze drier for 48 hours. Pathan et al., (2010) found that this method produced an acceptable preservation of bean (non-waxy) and wheat (waxy) samples. However it did dissolve waxes of other species like cabbage and broccoli.

Cryo-SEM is specialized and expensive equipment. However, it is a fast, consistent and an efficient way to overcome SEM preparation problems. Furthermore, this technique is extensively used to examine complicated and difficult samples such as those that exhibit beam sensitivity and those of an unstable nature. This method is also superior to the others as it reduces the amount of mechanical handling and mechanical damage to delicate samples. In addition, it eliminates the need of potentially damaging and harmful chemical fixatives. In this study, the best suited method for SEM preparation of *S. nicolai* was cyro-SEM. This method gave both acceptable image quality and minimal artefacts. Cryo-SEM revealed morphological details about fluid or air-filled spaces in plant tissue which are not readily apparent in fixed and dried tissue with conventional SEM (Jeffree et al., 1987).

Conclusion

The fixing of delicate plant tissue for scanning electron microscopy poses idiosyncratic intricacy. The results obtained in this study underpins preceding assessments that optimal SEM processing protocols fluctuate for dissimilar tissues. A choice of biological preparation techniques is applied to reduce problems stemming from delicate plant tissue characteristics and sample artefacts. These methods employed may reduce artefacts but no method, yet, can make certain of an artefact-free specimen (Pathan et al., 2009). In most cases delicate plant material have large vacuoles. This can cause the fixative concentrations to become dilute. Vacuoles collapse after the water is removed by dehydration (Hall and Hawes, 1991). As water makes up to 98% of some plant tissues, it causes a problem to most SEM preparation techniques. From this investigation of the preparation methodologies for the delicate aril tissue it can be concluded that the cyro-SEM preparation technique result in the best image quality because of its preservation of the seed aril tissue as close to its natural state as possible. It also has the added advantage of being less time consuming than the other methods. However, the drawback is that this method may be more costly in the long run due to the initial cost of the instrument and the large amount of liquid nitrogen used during each viewing session.

Acknowledgments

This research was supported by the National Research Foundation (NRF) of South Africa. We would also like to thank Mr Vishal Bharuth and Mr Subashen Naidu from the Microscopy and Microanalysis Unit at UKZN for their technical support and constant advice throughout this study.

References

1. Berlyn GP, Miksche JP. (1976). Botanical Microtechnique and Cytochemistry. Ames, Iowa.
2. Boesewinkel FD, Bouman F. (1984). The seed: structure. In *Embryology of Angiosperms* Springer-Verlag, Berlin. 565-610.
3. Boyde A, Boyde S. (1980). Further studies of specimen volume changes during processing for SEM: including some plant tissue. Scan Electron Microsc II:117–124.
4. Boyde A, Maconnachie E. (1979). Volume changes during preparation of mouse embryonic tissue for scanning electron microscopy, Scanning 2:149–163.
5. Boyde A, and Maconnachie, E. (1983). Not quite critical point drying. The Science of Biological Specimen Preparation for Microscopy and Microanalysis, Scanning Electron Microscopy Inc., Chicago.
6. Callow JA, Osborne MP, Callow ME, Baker F and Donald AM. (2003). Use of environmental scanning electron microscopy to image the spore adhesive of the marine alga Enteromorpha in its natural hydrated state. *Colloids Surf B Biointerfaces* 27, 315–321.
7. Hall JL. and Hawes C. (eds.) (1991). Electron Microscopy of Plant Cells, Academic, London, England.
8. Hardy JP, Anderson VJ and John and Gardner. 1995. Stomatal Characteristics, Conductance Ratios, and Drought-Induced Leaf Modifications of Semiarid Grassland Species .*American Journal of Botany.* 82, 1: 1-7.
9. Http://research.jcu.edu.au/enabling/aac/cairns-aac/biological-sample-preparation-for-the-sem. James Cook University, Australia (acsessed 15-02-2016).
10. Http://www.mbc.edu/faculty/pdeeble/BIO329/Web/329Notes/EM%20Overview.htm Overview of electron microscopy, an outline. Drs. John Shields and Mark Farmer (acsessed 15-02-2016).
11. https://www.emsdiasum.com/microscopy/technical/datasheet/cryosem_adv.aspx. Cryogenic Specimen Preparation Principles, Technical Data Sheets (acsessed 15-02-2016).
12. Https://www.jic.ac.uk/microscopy/intro_EM.html. Norwich research park, Norwich, UK. (acsessed 15-02-2016).

13. Jeffree CE, Read ND, Smith JAC and Dale JE. (1987). Water droplets and ice deposits in leaf inter· cellular spaces: redistribution of water during cryofixation for scan ning electron microscopy. Planta 172, 20- 37.

14. Lapsley KG, Escher FE and Hoehn E. (1992). The Cellular Structure of Selected Apple Varieties *Food Structure* 11:4:6 http://digitalcommons.usu.edu/foodmicrostructure/vol11/iss4/6

15. Parsons E, Bole B, Hall DJ and Thomas DE. (1974). A comparative survey of techniques for preparing plant surfaces for the scanning electron microscope. *J Microsc* 101(1):59–75.

16. Pathan AK, Bondb J and Gaskina RE. (2009). Sample preparation for SEM of plant surfaces. *Electron microscopy* 12:1369 -7021.

17. Pirone, C. (2010). "Bilirubin: an Animal Pigment in the Zingiberales and Diverse Angiosperm Orders". FIU Electronic Theses and Dissertations. Paper 336. http://digitalcommons.fiu.edu/etd/336.

18. Stokes DJ. (2008). Principles and practice of variable pressure/environmental scanning electron microscopy. Royal Microscopical Society. John Wiley and sons.

19. Takao I and Hitoshi O. (1988). Drying Method of Biological Specimens for Scanning Electron Microscopy: The t-Butyl Alcohol Freeze-drying Method. Arch. Histol. Cytol, (51) 1:53-59.

20. Talbot MJ and White RG. (2013). Methanol fixation of plant tissue for Scanning Electron Microscopy improves preservation of tissue morphology and dimensions. Plant Methods 9: 36.

21. Tsou CH, Cheng PC, Tseng CM, Yen HJ, Fu YU, You TR and Walden DB. (2015). Anther development of maize (*Zea mays)* and long stamen rice (*Oryza longistaminata*) revealed by cryo-SEM, with foci on locular dehydration and pollen arrangement. Plant Reprod 28:47 doi: 10.1007/s00497-05-0257-3

Chapter 6

Journal article for publication 5:

A comparative microstructural study of the animal pigment, bilirubin, in seed arils of *Strelitzia nicolai*

Depika Dwarka[1*], Nelisha Murugan[2], Veneesha Thaver[3], Mickey Naidu[4] and Himansu Baijnath[5*]

[1,5]School of Biological Sciences, University of KwaZulu-Natal, Durban, South Africa. [2]Microscopy and Microanalysis Unit, University of KwaZulu-Natal, Durban, South Africa. [3]School of Laboratory Medicine and Medical sciences, Discipline of Physiology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa. [4]School of Health Sciences, Discipline of Pharmacology, University of KwaZulu-Natal, Westville Campus, Durban. South Africa.

Email: depika.dwarka@gmail.com

Key words: *Strelitzia nicolai*, seed arils, bilirubin, light microscopy, cryo-SEM, Hall's staining and transmission electron microscopy

Abstract

The unexpected finding of an animal pigment, bilirubin, in the arils of the plant *Strelitzia nicolai* Regel & Körn. has unlocked a vast amount of interest regarding bilirubin's formation, its ultimate function and its ultrastructure. The objectives of this study were to identify the structural components of seed arils of *S. nicolai* at a cellular level. The second objective was to compare the five different stages of aril maturation and additionally to attempt to elucidate bilirubin occurrence within the cells. This was accomplished by stereomicroscopical and light microscopical analysis which included arils that were stained with toluidine blue and a modified Hall's staining to detect the presence of bilirubin.

In addition, arils were fixed in 2% glutaraldehyde, secondary fixed in 2% buffered osmium tetroxide, ultra-thinly sectioned and viewed under the transmission electron microscope. Fresh arils were viewed using a Zeiss Ultra Plus FEGSEM with a Quorum PP3000T cryo chamber (cryo-SEM). The results from the light and transmission electron microscopy studies showed that the different stages of aril development contain a vast quantity of cellular contents. As the aril matures, the contents of the cells became denser. Also, arils became a great deal more structured and rigid as they matured. The most noticeable trait of these arils is the colour changes as they mature. It was elucidated that the colour changes of the arils were attributed to the production and accumulation within the cells of bilirubin found in the arils.

Introduction

The angiosperm family *Strelitziaceae* contains 3 Genera (*Phenakospermum, Ravenala* and *Strelitzia)* and 7 Species. They are medium to colossal trees that are monoecious with flowers aggregated in 'inflorescences'. These species are found in sub-tropical to tropical regions from South America to South Africa and Madagascar (Watson and Dallwitz, 1992).

S. nicolai, commonly known as the 'White Bird of Paradise', 'bush banana' or 'wild banana', contains leaves resembling those of the banana plant. The flowers of this plant are white and are formed in clusters which contain bluish-purple petals that resemble a "tongue". A large amount of clear liquid accumulates at the base of the petal and sepal within the large bract. This fluid mostly attracts wasps, honeybees and flies to the flower. *S. nicolai* is pollinated mainly by sunbirds that consume the nectar (Frost and Frost, 1981)**.** The fruit is a 3-lobed and dehiscent woody capsule. *S. nicolai* reproduces mainly by seed. The seed is conspicuous and contains arils. The seeds, when fully mature, are black with orange, woolly arils. These seeds are dispersed by birds and other animals that are attracted to the colourful arils.

In 2010, Florida International University researchers made a breakthrough discovery in finding bilirubin in the arils of *S. nicolai.* Pirone et al., (2010) confirmed through HPLC/ESI-MS/MS the presence of the only human pigment, bilirubin, in a plant and also concluded that bilirubin is not only present at a high concentration in the arils of *S. nicolai* but also serves as the major pigment and is consequently accountable for colour production.

In mammals, bilirubin is the oxidative product formed from the breakdown of haem. An individual with normal human metabolism generates 250–400 mg/day of bilirubin through the breakdown of these haemoproteins (Chowdhury et al., 1995). Bilirubin is an orange tetrapyrrole dicarboxylic acid that is extremely hydrophobic and is produced when microsomal haem oxygenase enzyme catalyses the oxidation of haem to a green tetrapyrrolic bile pigment, biliverdin, which is hydrophilic. Then, biliverdin is transformed to bilirubin by biliverdin reductase (McDonagh, 2001). Bilirubin is excreted from the body through the bile ducts of the liver, typically as the main component of bile. If bilirubin is not excreted, it accumulates in the body and leads to toxicity and pathologic chemistry of intracellular organelles and physiological processes.

Bilirubin has attracted the attention of scientists since the earlier periods. Its chemistry, metabolism and elimination have been studied thoroughly during the last two centuries as a model for the removal of insoluble organic anions from the liver (Chen and Chen., 1984). Most scientists have previously been concerned about bilirubin being a marker for liver disease as well as its accumulation and resultant toxicity in the body. Recent studies have indicated that bilirubin at a limited level in the body could have many beneficial effects (Iłżecka and Stelmasiak, 2003). Recent advances and case studies have indicated that bilirubin at slightly elevated levels in humans and animals can serve as a potent antioxidant (Llesuy and Tomaro, 1994). After the discovery of bilirubin in a plant, this has created a new exhilaration around bilirubin's function and conception in the human and plant kingdom. Breimer et al., (1995) discovered an inverse relationship between serum bilirubin levels and the risk of ischaemic coronary artery disease. Zucker et al., (2004) observed a large number of subjects in the United States and they revealed that the probability ratio for colorectal cancer is reduced to 0.295 in men and 0.186 in women per 1 mg/dL increment in serum bilirubin levels.

The initial test for bile pigments was based on the assay by Leopold Gmelin (1788-1853) to detect bilirubin in urine. This reaction formed transient colours (green, blue, violet, red and yellow) during oxidation by concentrated nitric acid. An additional method was devised in 1917 by André Fouchet. This method was adapted and modified by M. J. Hall in 1960 and is currently still the most reliable and reproducible staining procedure for identifying bile pigments (Kiernan, 2008). Hall's staining method traditionally contains two components: Fouchet's Reagent and Van Gieson's Solution. Fouchet's reagent comprises ferric chloride and trichloracetic acid which oxidizes bilirubin to green biliverdin. Tissues are then counterstained with Van Gieson's solution which contains a 0.1% acid fuchsin in saturated aqueous picric acid. This colours collagen fibres red and muscle tissue yellow (Hall, 1960).

This study set out to investigate the structure of *S. nicolai* arils through histochemical techniques i.e. Hall's and toluidine blue staining methods in light microscopy, cryo-SEM and TEM analysis. Surface structures could not be identified through conventional SEM techniques due to the delicate nature of the sample. Cryo-SEM, coupled with freeze-fracturing, proved to be the best technique to allow for the preservation of the integrity, architectural stability and erudition of the arils.

Materials and methods

S. nicolai seeds (Voucher specimen: Dwarka and Baijnath 1, Ward Herbarium UDW) were collected at different stages of development from plants at the University of KwaZulu-Natal, South Africa. Arils were removed carefully and subjected to pre-treatment according to the subsequent microscopical analysis. For the purpose of this study the seeds were categorised into five stages according to their size, colour, aril size, aril thickness and aril colour.

Stereomicroscopical analysis

Fresh seeds with arils attached, were collected and viewed using a Nikon AZ 100 stereo microscope attached to a high performance camera using 4X objevcctive and Z-stacking was performed in order to create a single sharp image as well as to increase the depth of focus using the NISD Elements D 3.0 software.

Light microscopy analysis

Freshly collected arils of different stages of development were separated permanently, mounted on slides using Eukitt mounting medium and viewed using a Nikon Eclipse 80i. Arils were free hand-sectioned for light microscopy. Arils were viewed unstained or stained with a modified Hall's stain and also toluidine blue.

Modified Hall's staining

A modified Hall's staining method was used. Fresh arils were free-hand sectioned to view the cross sections using a Minora double edged blade. Sections were stained in freshly prepared Fouchet's solution (Trichloroacetic acid, 10% Ferric chloride & distilled water) for 15 minutes. Thereafter, the slides were rinsed in water, followed by distilled water, allowed to completely dry and then

permanently mounted using Eukitt mounting medium. According to the Hall's method (1960), Fouchet's solution stains bilirubin globules olive green/emerald green.

Cross and longitudinal sections of arils stained with toluidine blue

Individual arils at different stages of development were fixed in 2% glutaraldehyde and the presiding steps in the protocol were identical to that of TEM. Subsequent to being sectioned, and mounted on glass slides, samples were placed in 1% toluidine blue for 1 minute, rinsed in water for 1 minute and allowed to air dry.

Cryo-SEM

Fresh aril tissue was mounted on a specimen block using a colloidal graphite paste. Once the aril tissue was in position, it was plunged into nitrogen slush in a Quorum PP3000T chamber. Once frozen, the whole unit was transferred into the cryo-preparation chamber of the SEM at a temperature of 90°C and a pressure of 1.3 X 10^3 Pa (high vacuum conditions). After approximately 5 min (sublimation stage), the sample was sputter coated with 8 nm platinum. Thereafter, the sample was transferred under vacuum onto the cooled stage of the Zeiss Ultra Plus FEGSEM.

TEM Embedding

Fresh arils were fixed in 2% buffered gluteraldehyde for three days. Arils were then washed with 0.05 M sodium cacodylate buffer twice for 30 min each. After washing, the arils were secondary fixed in 2% buffered osmium tetroxide, overnight. Thereafter, samples were rinsed twice in sodium cacodylate buffer for 30 minutes each and then dehydrated through a graded ethanol series (10% ethanol through to 100% for 2 x 10 minutes). Thereafter, arils were transferred to propylene oxide solution for 15 min, and allowed to infiltrate overnight in a 50:50 mixture of Spurr resin: propylene oxide. Lastly, the samples were submerged in freshly made 100% Spurr resin and were then polymerized in an oven at 70°C for 24 hours. Following polymerization, the samples were sectioned using a Leica EM UC 7 microtome and images were captured using a Jeol 1010 TEM microscope.

Results

Figure 1 shows *S. nicolai* arils attached to the seeds at different stages of development as classified in this study. In stage 1, the seed is small with short semi-translucent arils. At stage 2 the seeds increase in size as the endosperm enlarges. The resulting arils are long and white compared with stage 1. The

aril colour turns from white to yellow, as seen in stage 3. At stage 4 the seed is much firmer than the earlier softer stages and the colour of the aril turns orange. In stage 5, which is the final stage, the seed is much smaller than the seeds from the fourth stage arils. The seed also turns black with a very tough seed coat and the arils are deep orange.

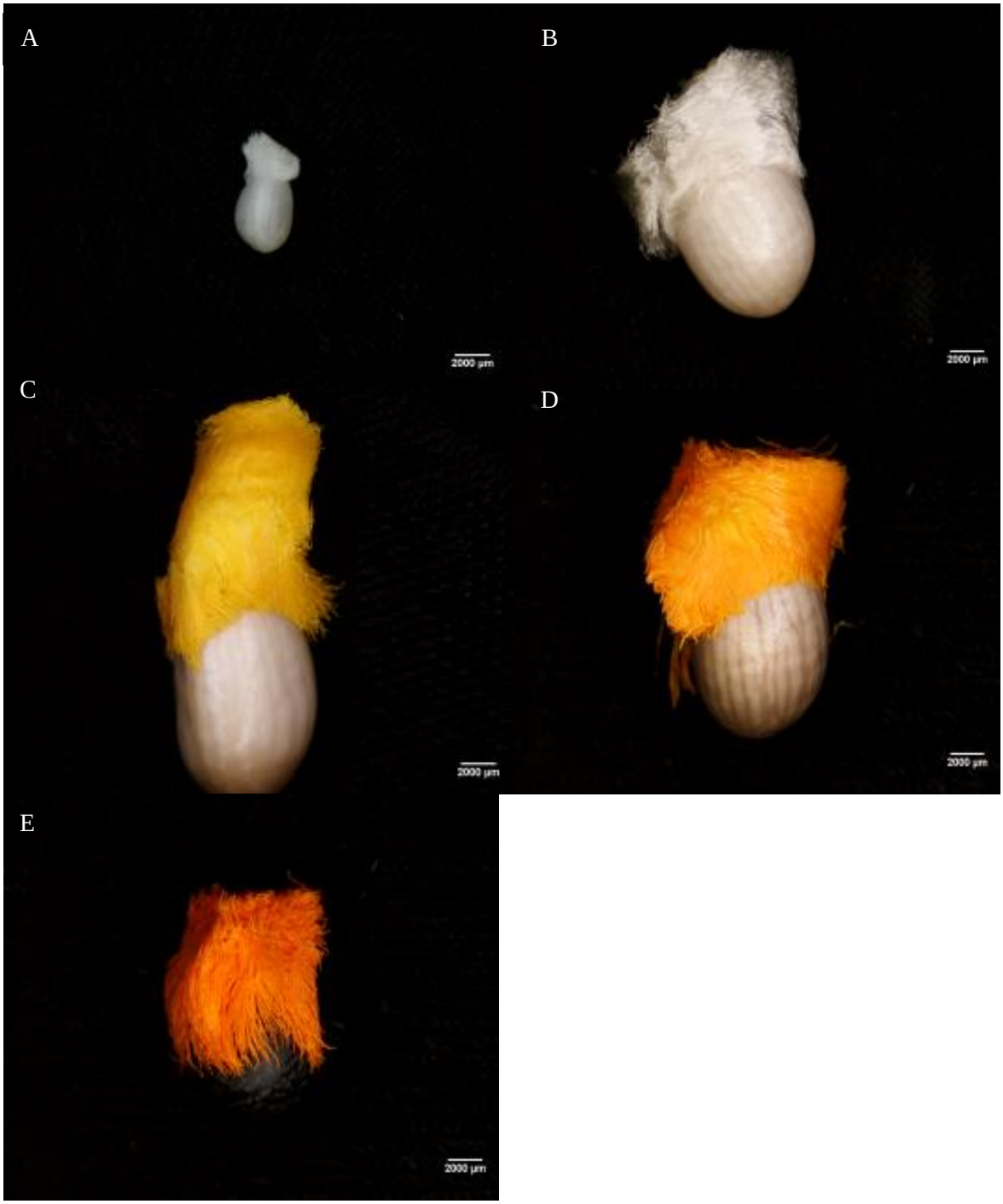


Figure 1: Stereo micrographs of the 5 different stages of aril development in seeds of *S. nicolai* as categorised in this study, showing (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5 arils attached to the seeds.

The aril colour, size and thickness also change through its development (Figure 2). Initial observation made of arils in the early stages of development seems to elude masses of liquids similar to that of water. In later stages, more mature stages (from stage 3-5), these liquids are noted to be analogous to oil.

Figure 2: Z-stacked micrographs (4X magnification) of the different stages of aril development showing (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5 arils.

Figure 3 shows the unstained individual arils as they develop and undergo the colour change. Figure 4 shows arils that were stained with the modified Hall's staining. In the early stages the stain is not fixed to the arils. As the aril matures, the stain becomes more apparent and denser in selected areas.

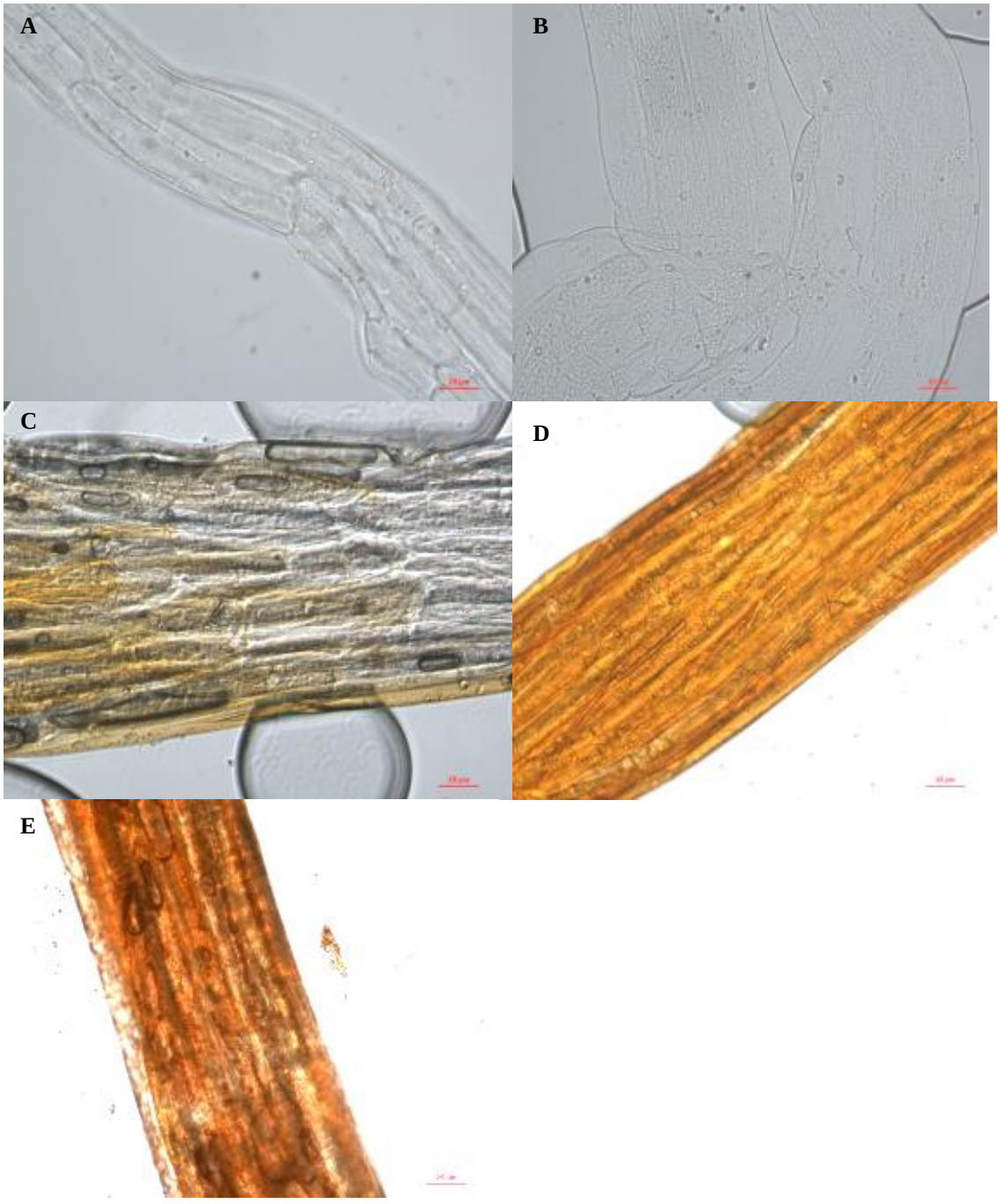


Figure 3: Light micrographs of individual unstained arils at the 5 different stages of development showing (A) stage 1, (B) stage 2, (C) stage 3, (D) stage 4 and (E) stage 5

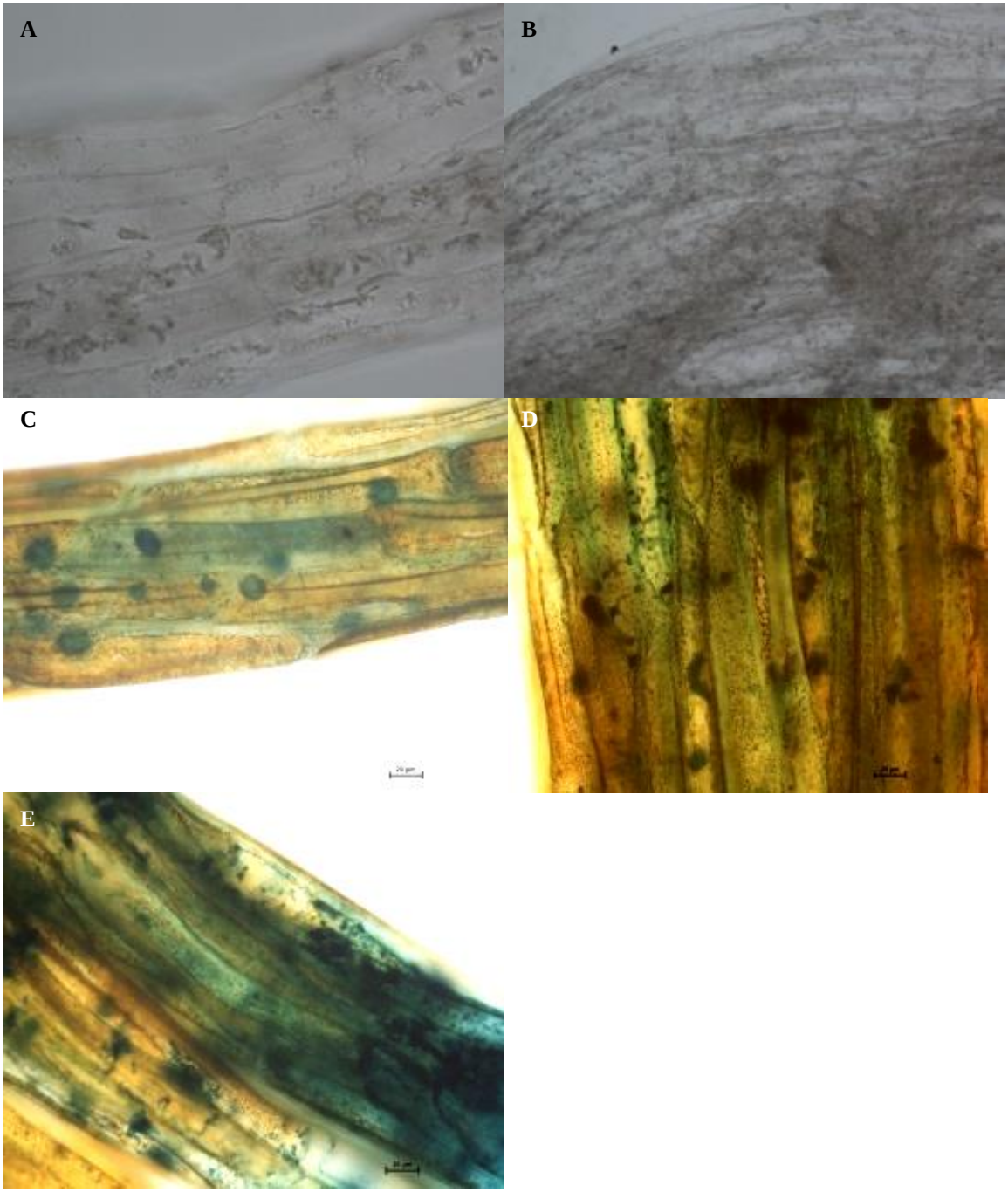
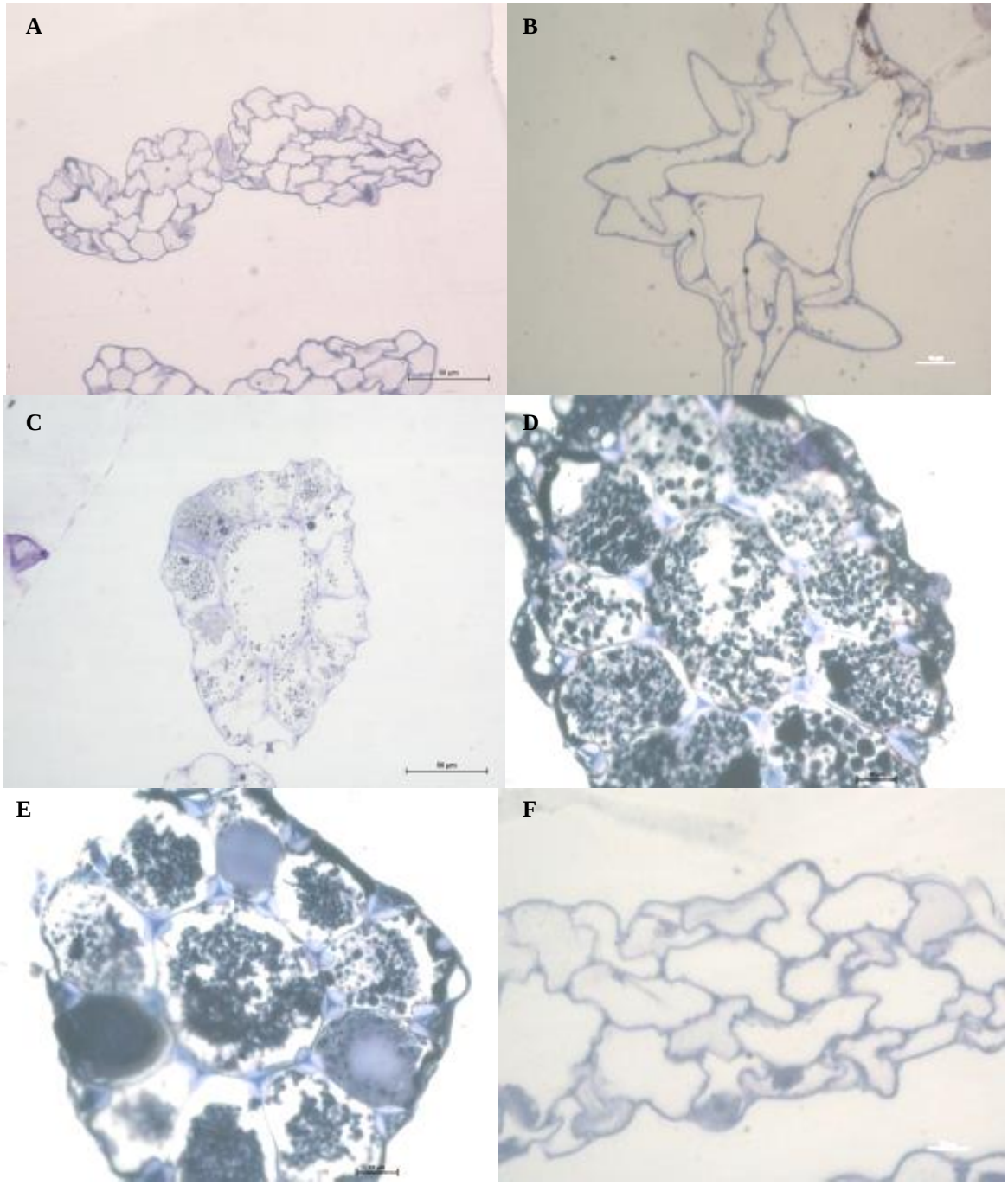


Figure 4: Free hand sections of the arils (stages 1-5) stained with the Hall's stain, (A) represents stage 1, (B) shows stage 2, (C) stage 3, (D) stage 4 and (E) stage 5 arils

Figure 5 shows arils that were fixed in 2,5% glutaraldehyde and were sectioned and stained with toluidine blue. As shown in the Figure 5, the early stages (1 and 2) appear to be immature and the overall configuration is quite unstructured. From stage 3 onwards, the arils are more defined and rigid. This was observed in both the cross section (Figure 5 A-E), as in well as the longitudinal sections

(Figure 5 F-J). As the arils matured, they became more pigmented, as seen in Figure 5 where toluidine blue stained pigments darker blue as the aril development advanced.



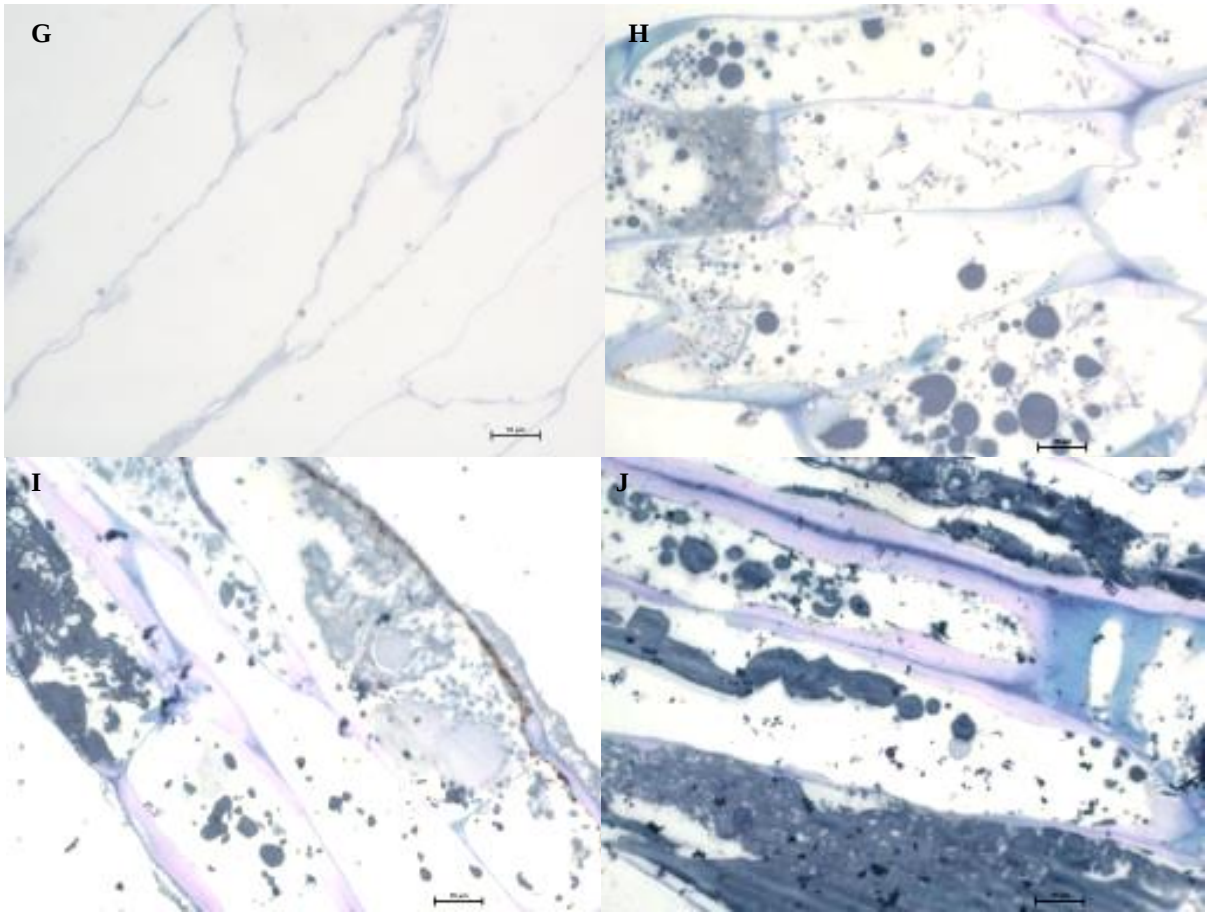


Figure 5: Cross (A-E) and longitudinal sections (F-J) of *S. nicolai* arils stained with toluidine blue. As the stage progresses the content of the cells becomes denser.

Figures 6-10 show cryo-SEM images of the arils. These show surface structures as well as internal structures when fractured at cross sections and longitudinal sections. At stage one, the aril (6A) seems to be very rigid when observed. However, when it is fractured the internal organelle seems poorly developed. Stage one contains masses of liquid similar to that of water. Therefore, when the external structure plunged into liquid nitrogen it was well preserved. Going on to stage 2, the external (7A) as well as internal (7B and C) seems to be more developed. The poorly developed stage one is now more structured internally.

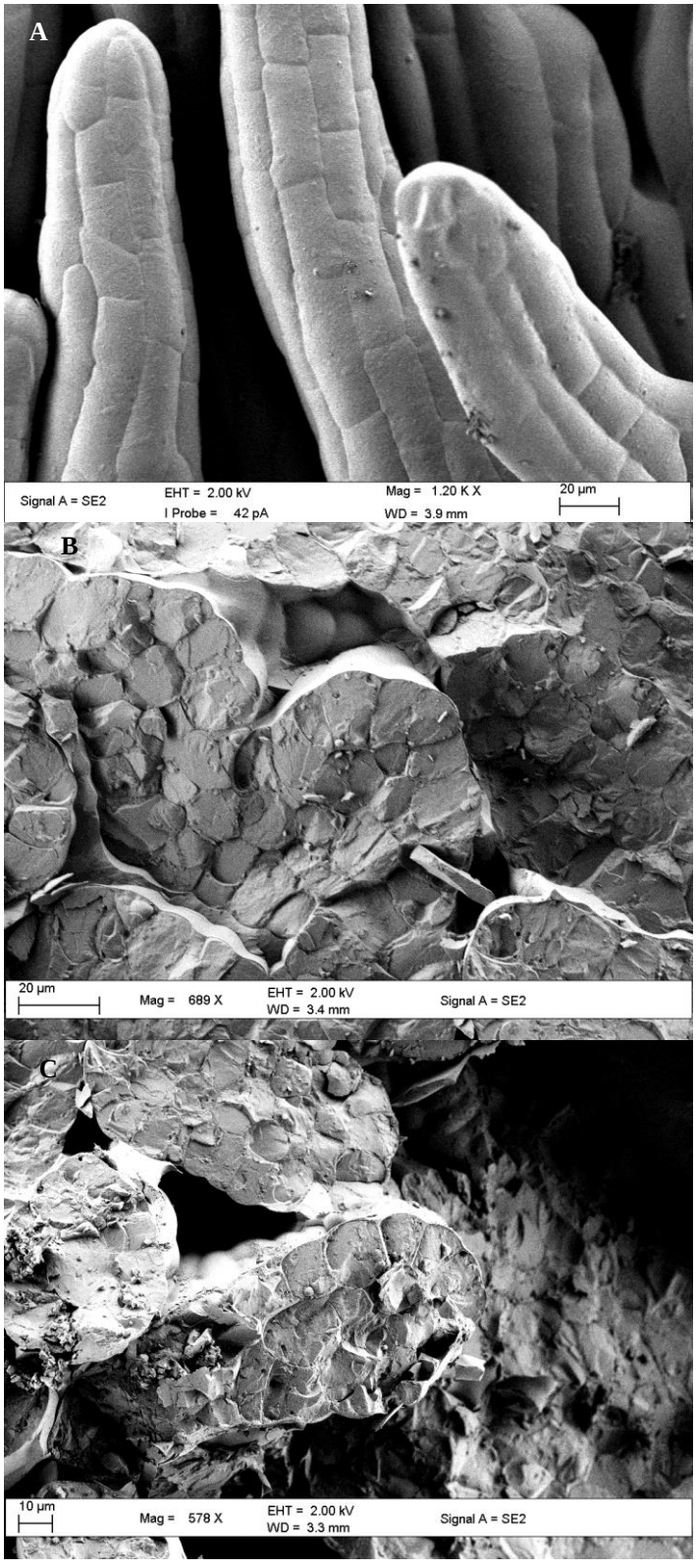


Figure 6: Stage 1 cryo-SEM micrographs of (A) surface, (B) cross section fracture (C) and longitudinal fracture of arils of *S. nicolai*. This demonstrates the lack of structural components in the initial stage.

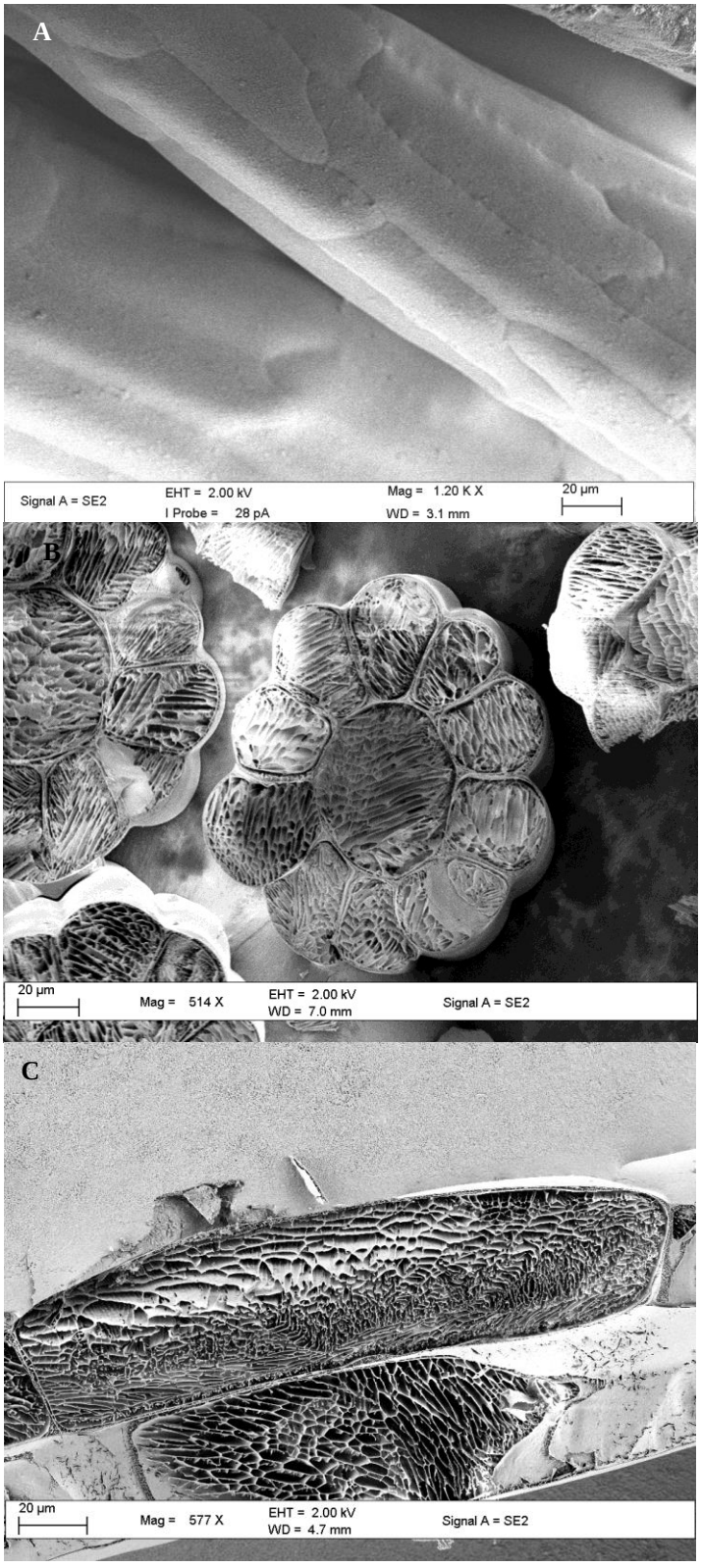


Figure 7: Stage 2 cryo-SEM micrographs of (A) surface, (B) cross section fracture and (C) longitudinal fracture showing a cylindrical and lobed structure.

Figures 8-10 show stages 3-5 of the arils external and internal structures. From stage three (Figure 8) onwards, the appearance of small, spherical bodies (arrows) that encompass the arils was noted. At stage 4 (Figure 9), the circular bodies become more intense and also the appearance of globules (arrows) on the surface of the arils are noted. At stage 5 (Figure 10), the surface globules become more conspicuous and the internal circular globules seem to be caving in.

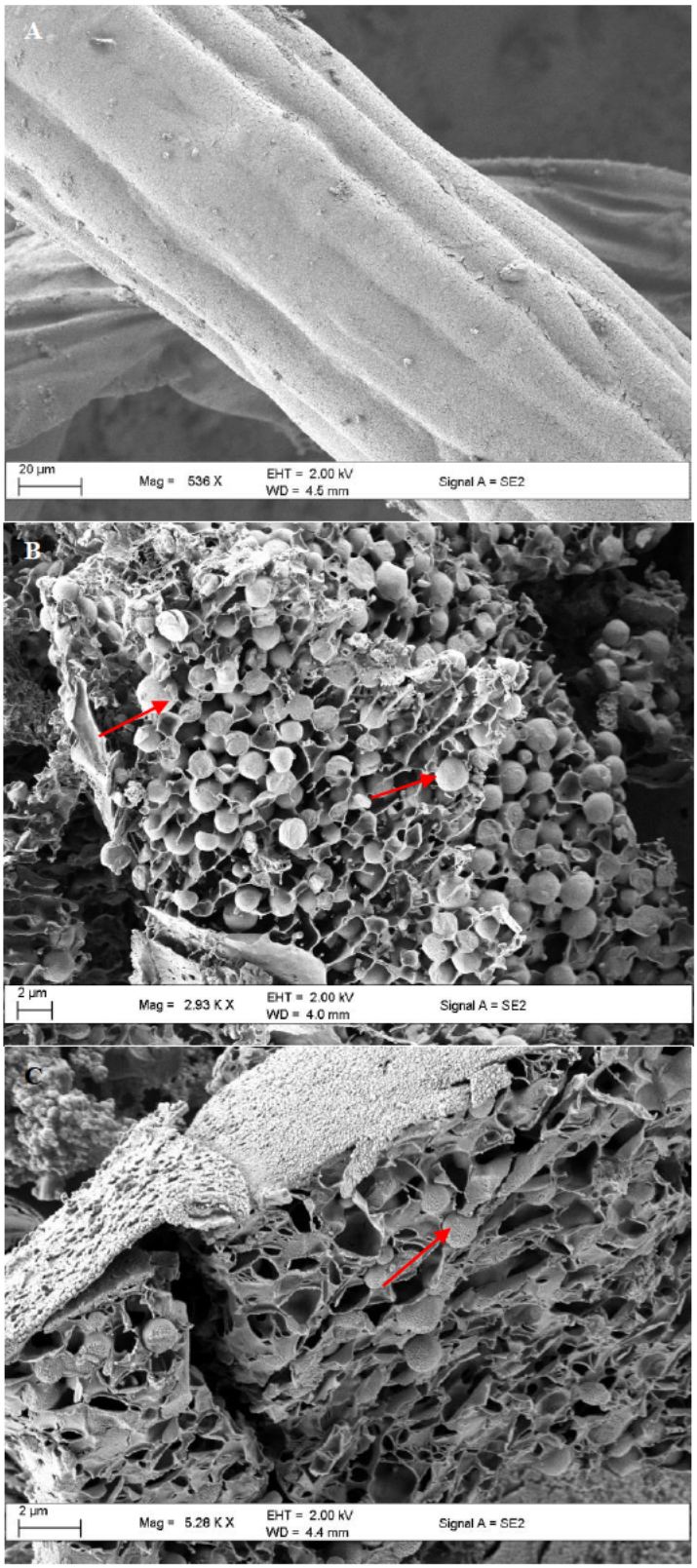


Figure 8: Stage 3 cryo-SEM micrographs of (A) surface, (B) cross section fracture and (C) longitudinal fracture of arils. Arils increased in length and thickness and therefore became additionally structured in stage 3. Arrows indicate what appears to be the formation of bilirubin.

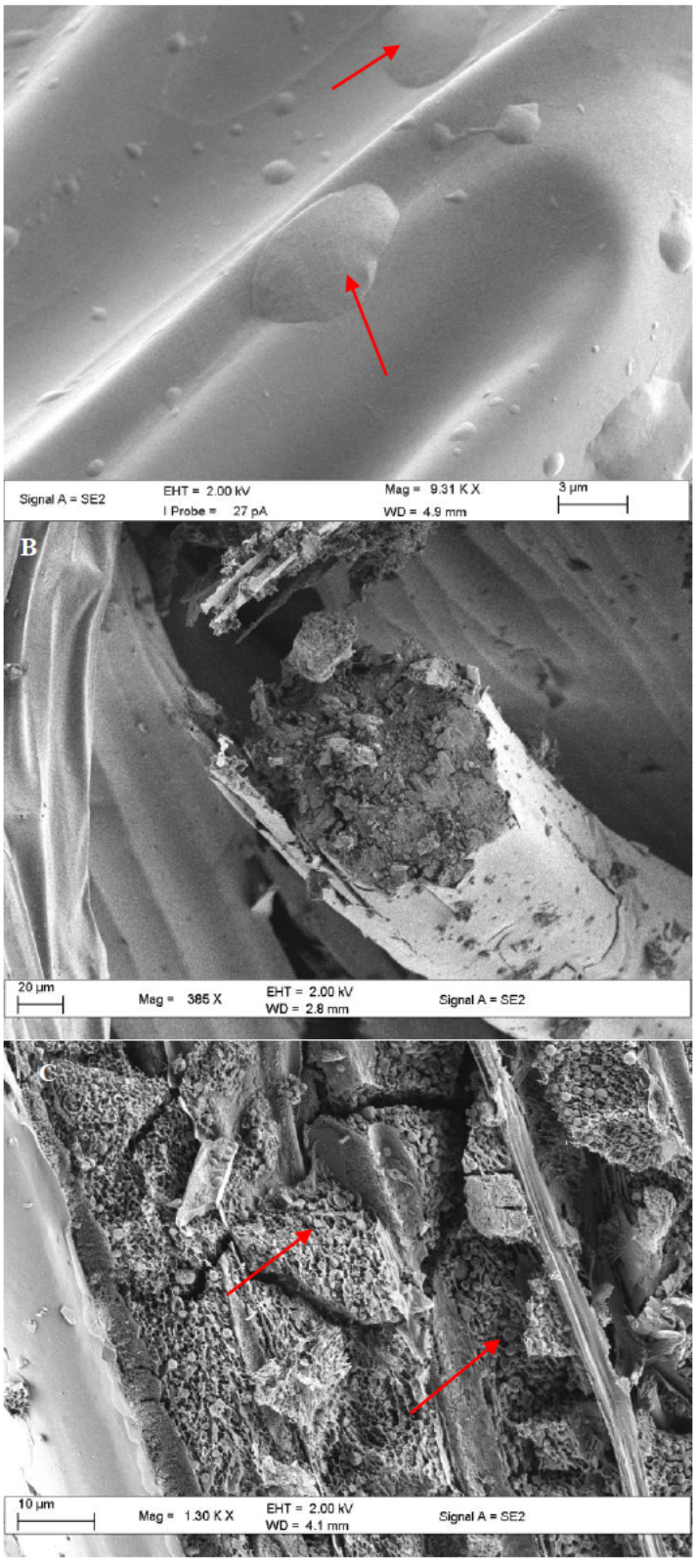


Figure 9: Stage 4 cryo-SEM micrographs of (A) surface showing what appears to be bilirubin on the surface (indicated with arrows), (B) cross section fracture showing accumulation of the pigment and (C) longitudinal fracture showing the partial dilapidation of arils possibly caused by the accumulation of what appear to be bilirubin.

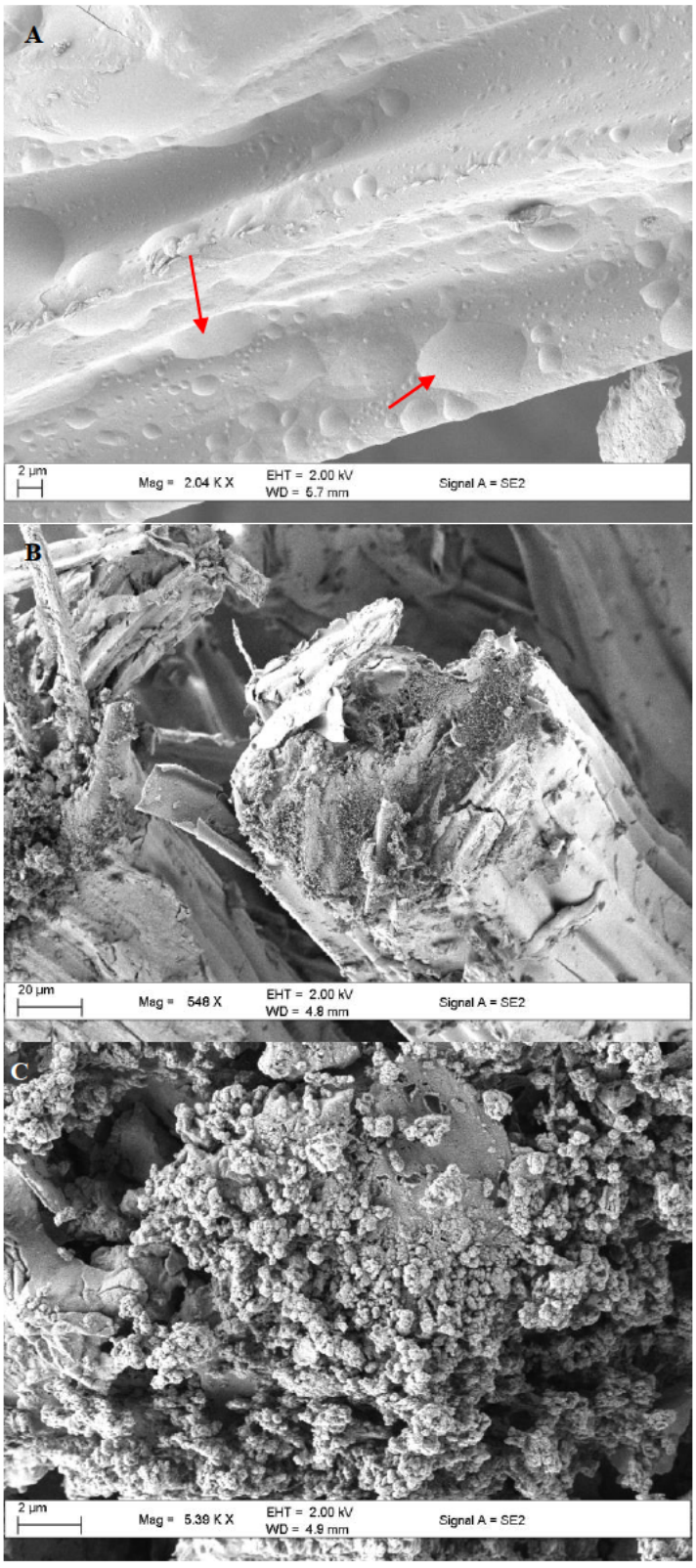
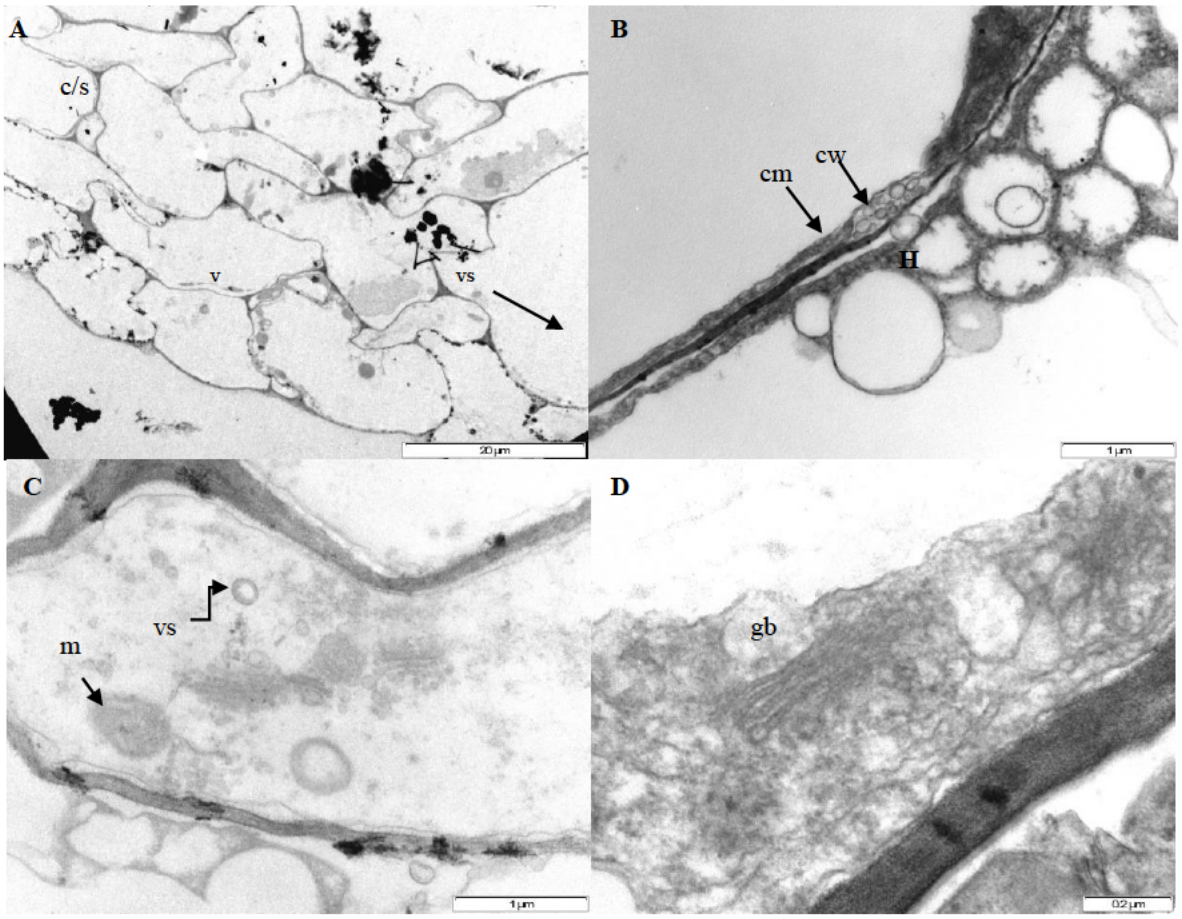


Figure 10: Stage 5 cryo-SEM micrograph of (A) surface, (B) cross section fracture and (C) longitudinal fracture of arils of *S. nicolai*. Figure 10A shows the accumulation of artefacts on the surface, this is a great deal more than what was observed in the previous stages. Figures 10B and 10C show cells that are completely filled with particles thought to be bilirubin.

Figure 11 shows the TEM images of different organelles found in the cross section and longitudinal section of stage 1 arils of *S. nicolai*. Figure 11A shows an overall cross section of stage 1. Stage 1 contains a large number of nuclei, mitochondria, endoplasmic reticulum and vesicles. This suggests that this tissue is metabolically active.



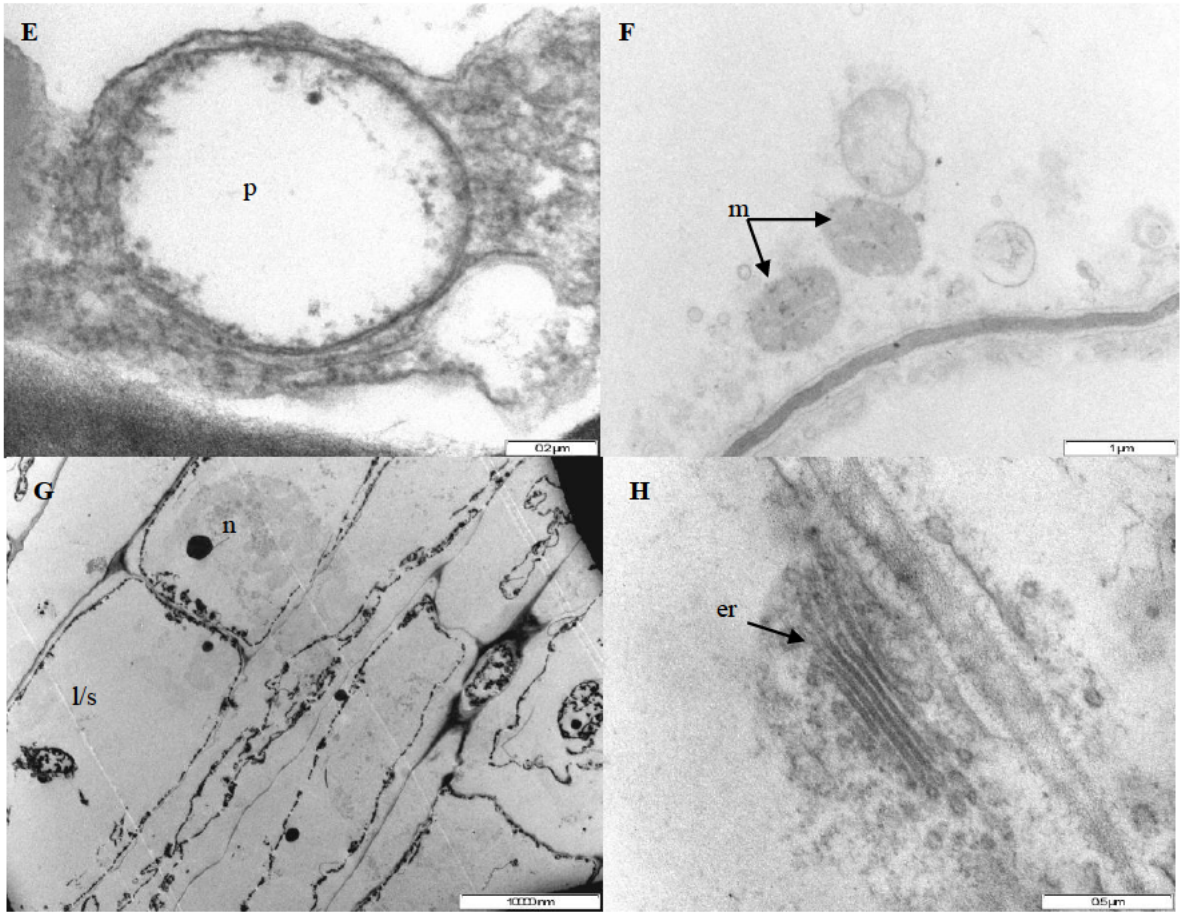


Figure 11: TEM micrographs of stage 1 arils (A-F) and longitudinal section (G and H) of arils of *S. nicolai*. (A) shows cross section (c/s) of aril, (B) cell wall (cw) and cell membrane (cm), (C) arrows show mitochondria (m) and vesicles (vs), (D) golgi body (gb), (E) vacuole (v) and large plastid (p), (F) mitochondria (m) and vesicles (vs), (G) longitudinal section (l/s) of aril showing nucleus (n) and (H) showing endoplasmic reticulum (er).

Figure 12 illustrates stage 2 arils that were sectioned for TEM. Figure 12A shows an overall cross section of with cells that are poorly developed. During stage 2 the cytoplasm becomes to some extent granular, in which plastids occur. Figure 12C illustrates noticeably developed plastids containing starch grains.

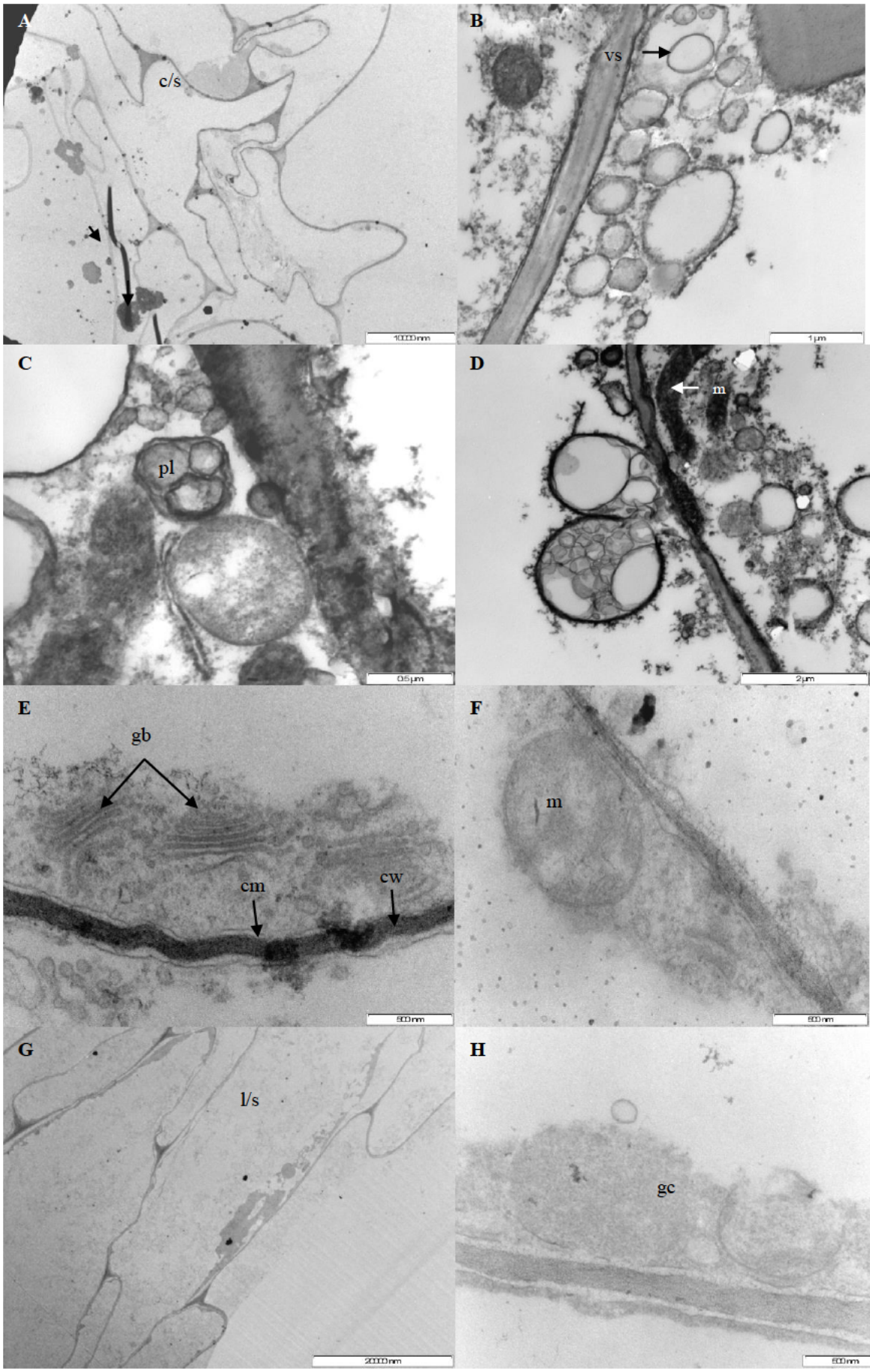
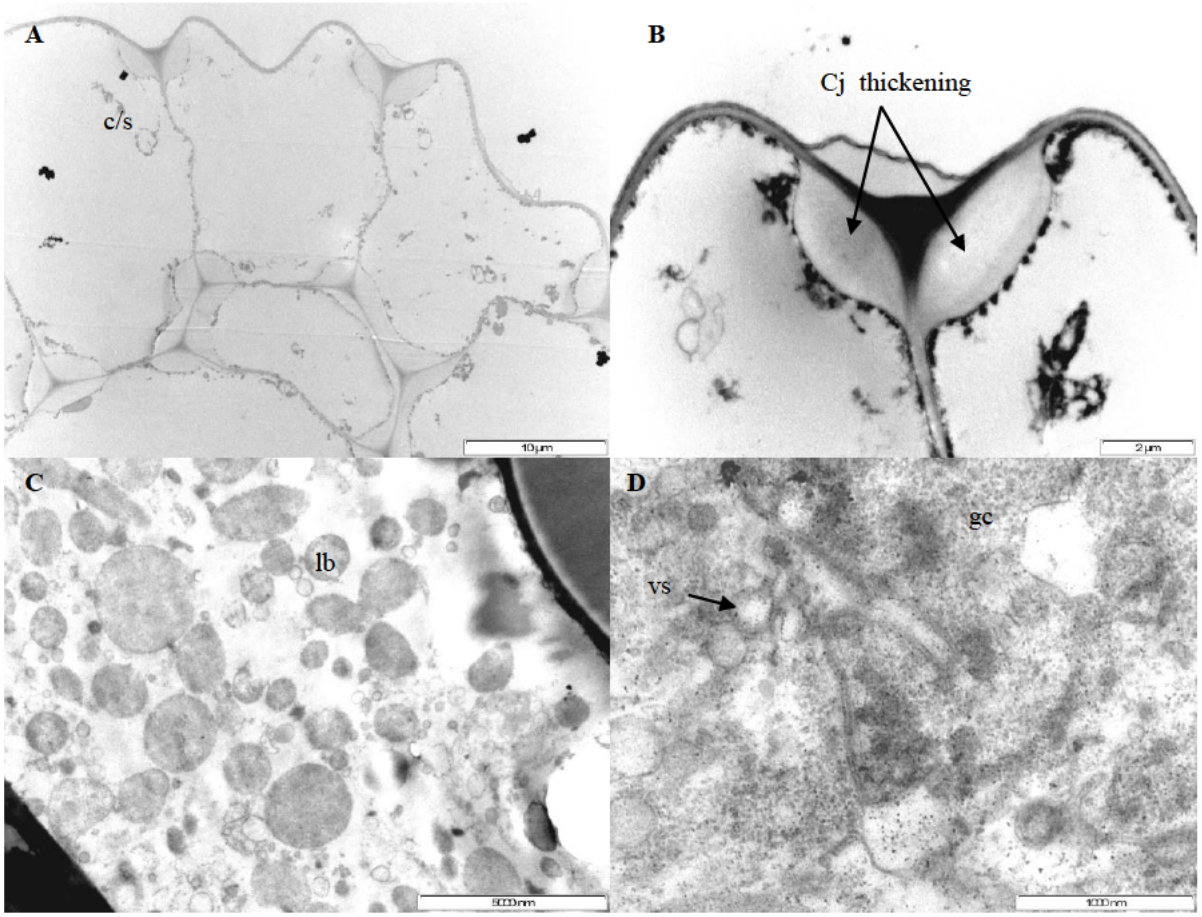

A
c/s
B
vs
C
pl
D
m
E
gb
cm
cw
F
m
G
l/s
H
gc

Figure 12: TEM micrographs of stage 2 cross section (A-F) and longitudinal section (G and H) of arils of *S. nicolai*. (A) shows cross section of aril (c/s), (B) vesicles (vs), (C) plastid (pl) containing starch grains, (D) elongated mitochondria (m), (E) golgi bodies (gb) and cell wall (cw) together with cell membrane (cm), (F) mitochondria (m), (G) shows longitudinal section (l/s) of aril and (H) granulated cytoplasm (gc).

Figure 13 are TEM images of stage 3 arils fixed and sectioned. Observed in Figure 13B is the cell junction that is starting to thicken from this stage onwards. Figure 13C shows numerous lipid bodies that began to form. The cytoplasm that was granular starts to become more or less coarse as opposed to the cytoplasm in stage 2.



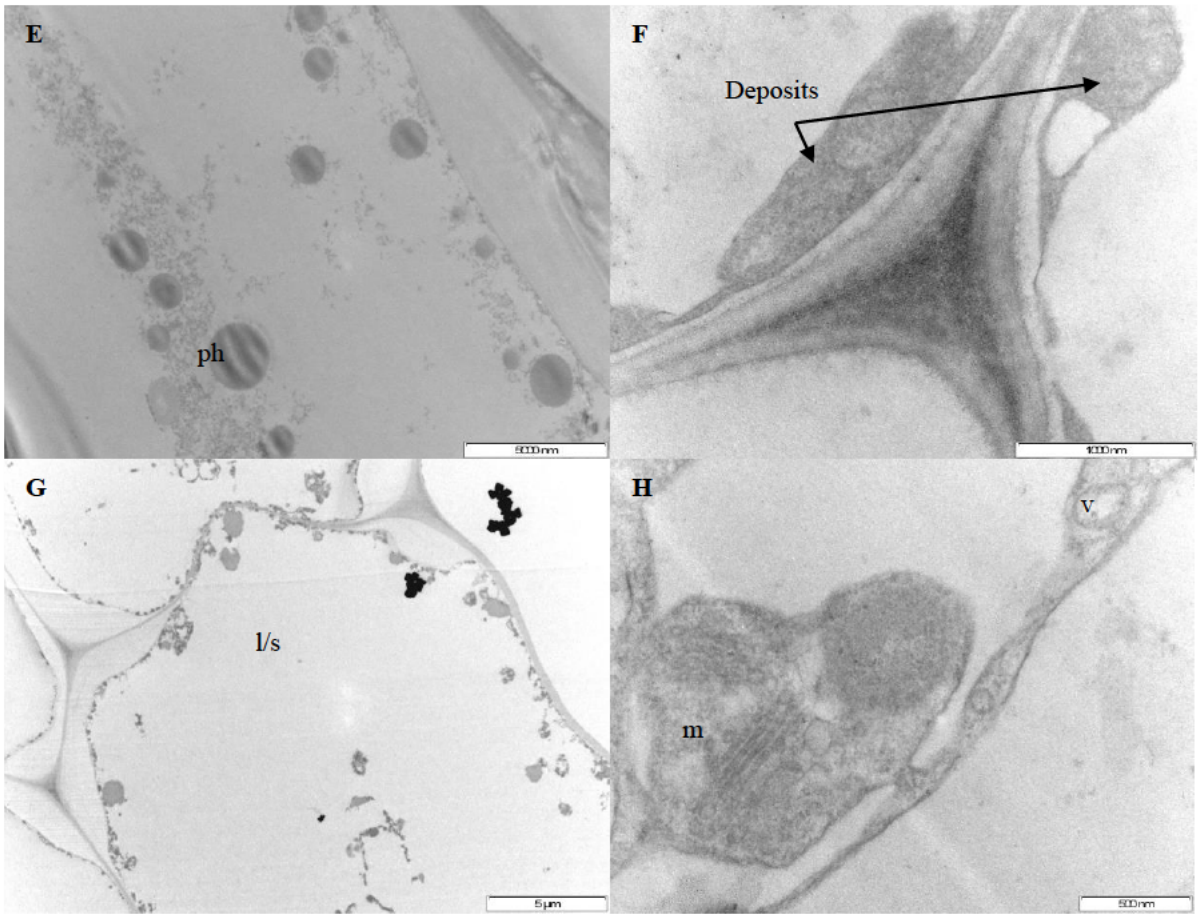


Figure 13: TEM micrographs of stage 3 of cross section (A-F) and longitudinal section (G and H) of arils of *S. nicolai*. (A) shows cross section (c/s) of aril, (B) cell junction (cj) thickening, (C) lipid bodies (lb), (D) granular cytoplasm (gc) that has become dense, (E) phenolics (ph), (F) deposits form cell junction (cj), (G) shows longitudinal section (l/s) of arils and (H) swollen mitochondria (m).

Figure 14 illustrates TEM images of stage 4 arils with apparent pigmentation and lipids. Stage 4 cross and longitudinal sections also show the cytoplasm that was granular has turned moderately globular due to the increased amount of lipid.

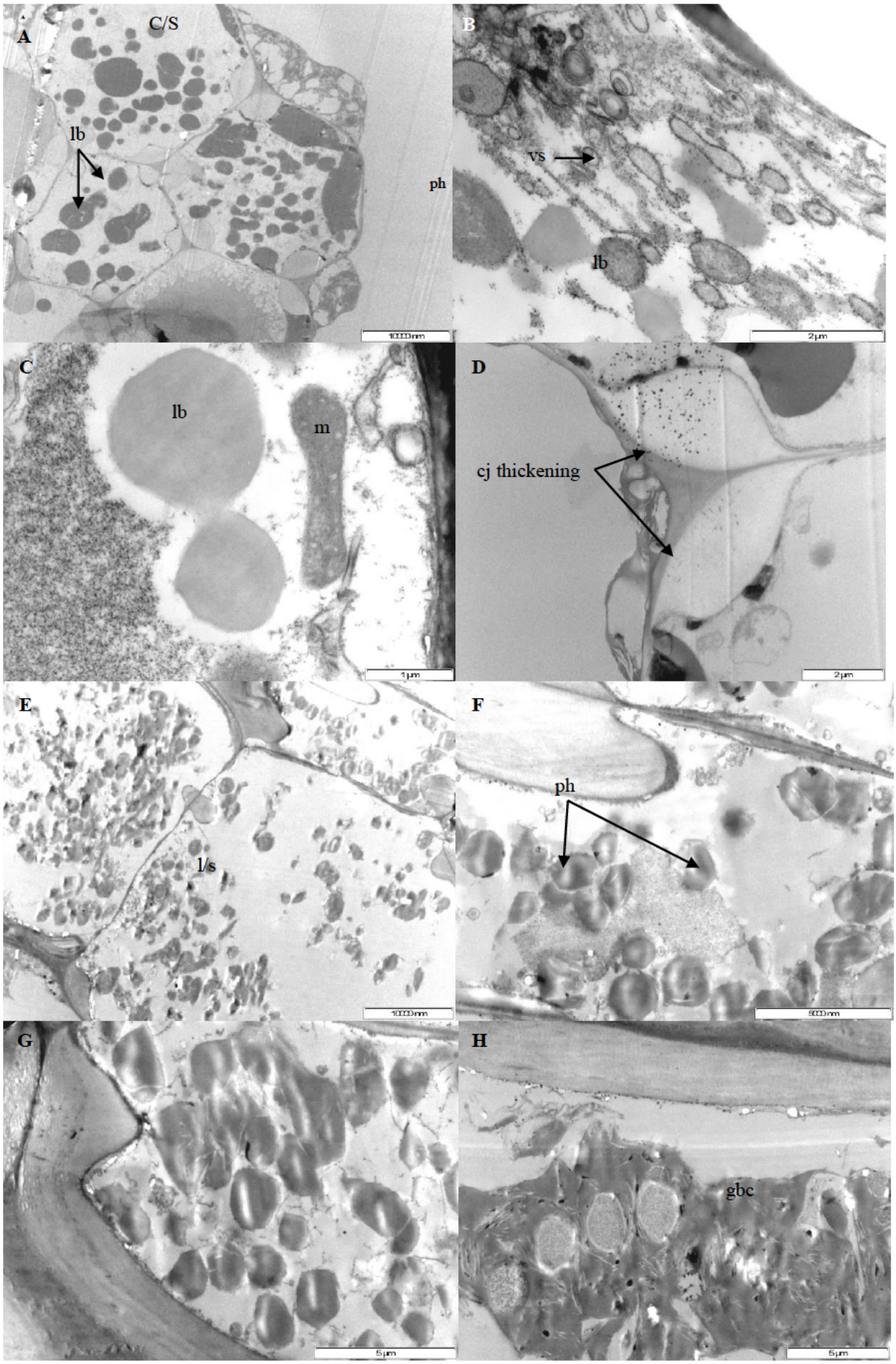
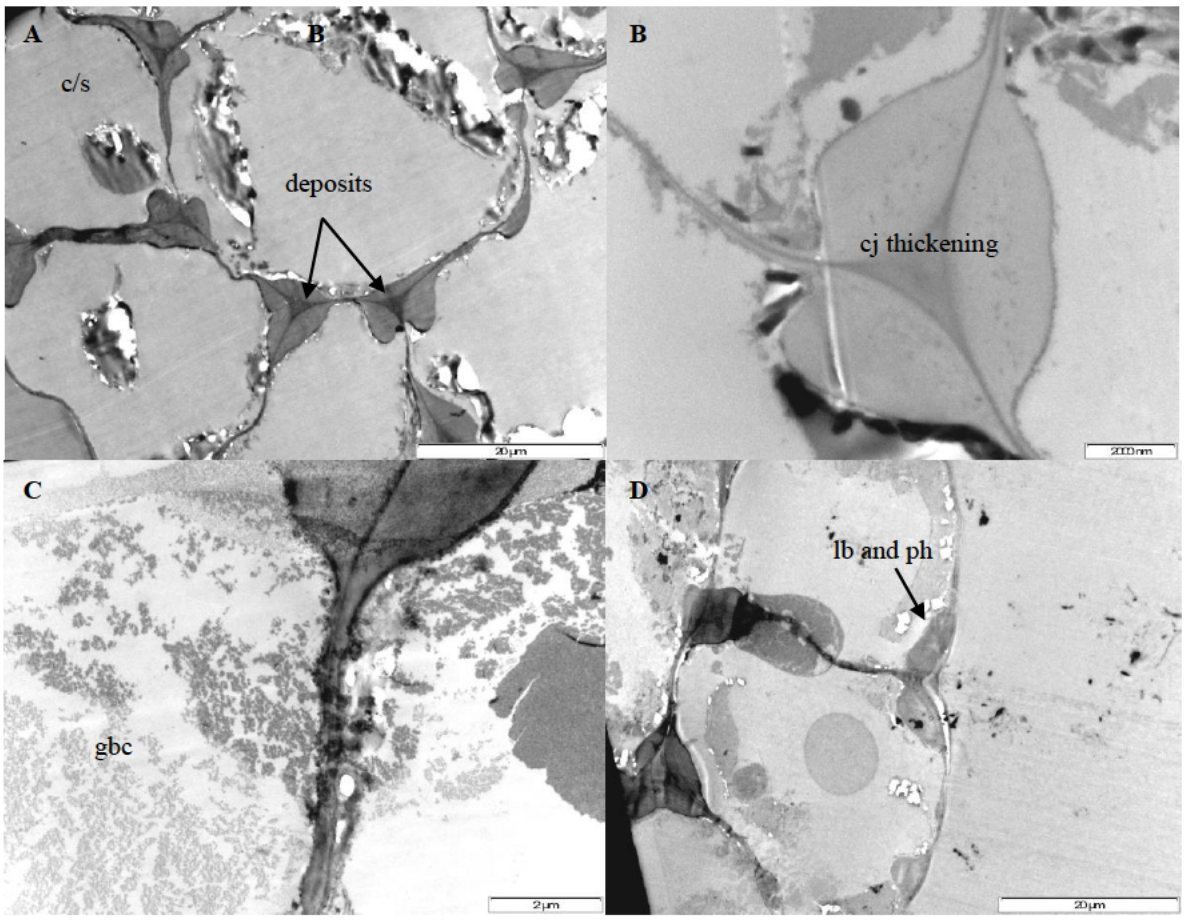

A
C/S
lb
ph
B
vs
lb
2µm
C
lb
m
1µm
D
cj thickening
2µm
E
l/s
F
ph
G
5µm
H
gbc
5µm

Figure 14: TEM micrographs of stage 4 of cross section (A-D) and longitudinal section (E-H) of *S. nicolai* seed arils. (A) shows cross section (c/s) of aril, 9B) lipid bodies (lb) and vesicles (vs), (C) larger lipid (lb) bodies and mitochondria (m) with a loss of internal structures, (D) cell junction (cj) thickening, (E) shows longitudinal section (l/s) of seed aril, (F) phenolics (ph), (G) more phenolics (ph) and (H) globular cytoplasm (gbc) that appears oily.

Figure 15 illustrates stage 5 arils that were fixed in glutaraldehyde and sectioned for TEM. The cell junctions with thickening are undoubtedly observable at low (Figure 15A and 15C) and high (Figure 15B) magnification. Figure 15F and 15G shows the globular cytoplasm which is believed to be linked to bilirubin.



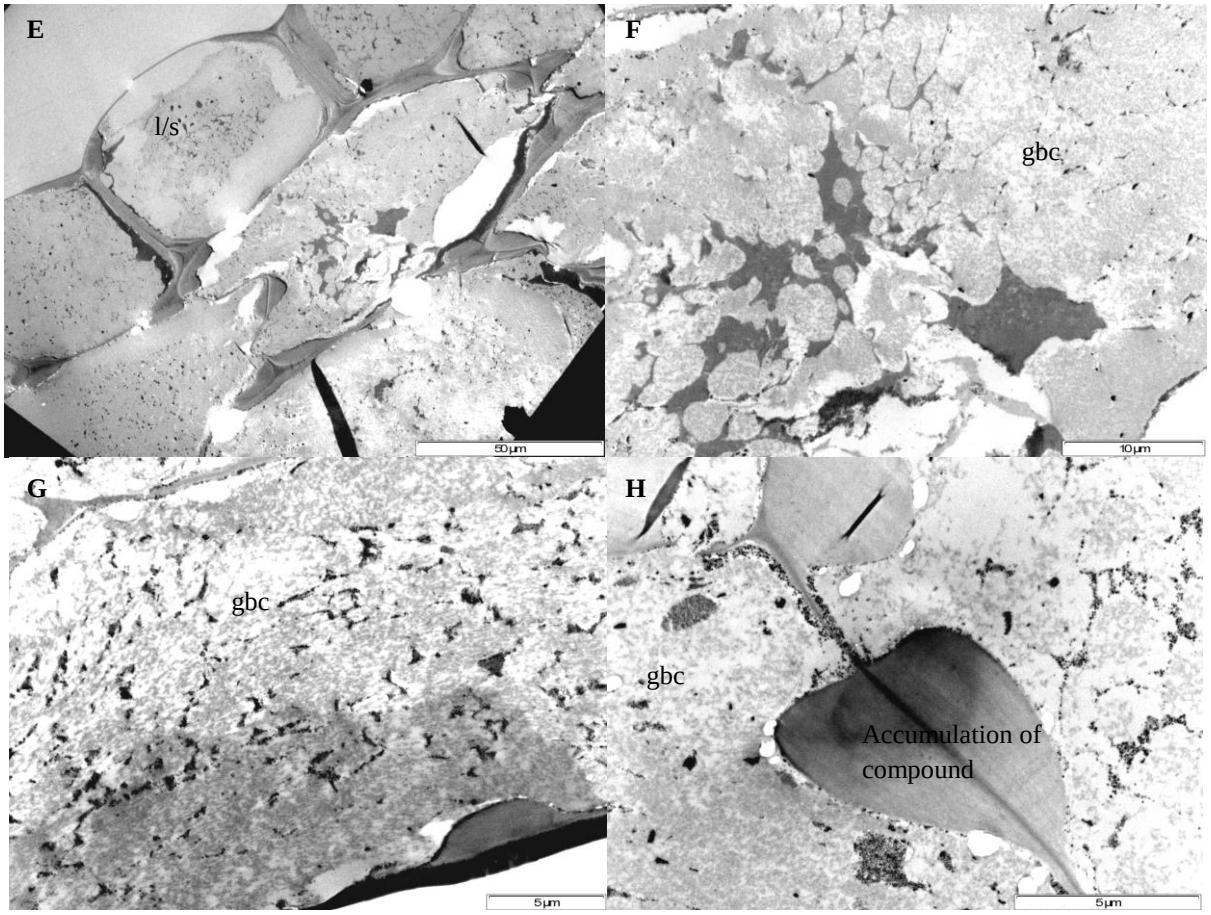


Figure 15: Stage 5 TEM micrographs of cross section (A-D) and longitudinal section (E-H) of arils of *S. nicolai*. (A) shows cross section (c/s) of aril illustrating deposits, (B) cell junction (cj) thickening, (C) globular cytoplasm (gbc) that seems to be oily in nature, (D) lipid (lb) and phenolic (ph) deposits, (E) shows longitudinal section (l/s), (F) globular cytoplasm (gbc) containing oils, (G) shows more of the globular cytoplasm (gbc) and (H) shows accumulation of compound.

Discussion

In this study, five different stages of *S. nicolai* aril development were categorised and their morphological and ultrastructural changes were studied. The arils were categorised according to the colour changes in the seed arils from opaque, white, yellow and light orange just before turning deep orange. This pattern is uniquely noticeable as the seed arils underwent the different stages of seed maturity. The aril length as well as the aril thickness also increases as the seed reaches maturity. The texture of each aril seems to be fairly woolly but enormously delicate and flimsy at the earlier stages and a much firmer at the later stages. Carbajose and Carbonero, (2005) showed that seed maturity guides a developmental end-point in the endosperm. Both embryo and endosperm display many

characters relating to the physiological transformation and underlying molecular methods linked with maturation. Similarly, aril maturity follows this sort of tendency.

The trichloroacetic acid and ferric chloride present in Fouchet's solution converts the orange colour of bilirubin to green biliverdin by oxidation (Figure 4). This stain is very specific to bile components and gives impervious results. In order to detect bilirubin in the arils, the Hall's staining had to be modified. One of the main challenges was that fixed aril tissue did not allow for the penetration of the stain. Therefore, fresh material had to be used. When arils were stained with the Hall's stain (Figure 4), from stage 3 onwards, there was a noticeable green pigment that was observed. It is proposed that the arils stained positive for bilirubin, similar to that of the liver tissue in humans.

The cryo-SEM images showed the trend of poorly developed arils at the initial stage of development then becoming more structured from stages 3 onwards. The internal structure appears to have had a plate like structure that was arranged radially in an exceptionally controlled approach. From stage 3 onwards there was an increase in the amount of pigment which was assumed to be bilirubin. On and after stage 3 the well organised crystalline structure started to collapse and became more unsystematic. Qian et al., (2013) reported this kind of structure in pigment stones. They reported clump like and sphere like bilirubinate particles in these stones. In addition, from stage 3 onwards, the water like consistancy of the liquid that was observed in the cells was replaced with lipid bodies together with bilirubinate bodies, therefore causing oil like consistency of arils at later stages. As the arils mature to stage 4 and 5 the amount of lipid and bilirubin also increases. This explains the colour changes of the arils from the initial to mature stages. Fortuna, (1982) confirmed that aril augmentation also follows a sigmoid curve. The mature aril is striking to fruit-eating birds and is non-hazardous, helping to endorse dispersion of the seed. The birds assimilate the fleshy aril as a food resource and pass the seed out in their excrements.

Toluidine blue is a polychromatic basic dye that stains acidic tissue components via metachromasia. Toluidine blue stains nuclei dark blue but will stain mast cell granules and polysaccharides violet. From stage 1 to 5 (Figure 5), toluidine stains aril cell walls purple, owing to the presence of polysaccharides. Initiating at stage 3, toluidine stains a few globules dark blue. Through to stage 4, the globules became more pronounced and aggregate together. By stage 5, the cells seem to be fully laden with dark blue staining. There's a good possibility that this darkblue colour could be staining the

bilirubin found in the cell. Bilirubin is a dicarboxylic acid (Broderson, 1986) and toluidine is a basic dye that has a high affinity for acidic compounds.

The TEM images demonstrated, that the early stages of maturity (Figure 11 and Figure 12) showed the presence of numerous mitochondria. The mitochondria found in stage one appeared larger than the ones found in stage two. The presence of numerous mitochondria suggests a high cell metabolism since mitochondria are responsible for cellular respiration. Gunning and Steer (1975), reported that the cell and mitochondria enlarge so the rate of respiration increases in proportion. From stage 3 onward the mitochondria became enlarged with the loss of the internal cristae structure. This could be attributed to the increase in bilirubin within the cell. Lakovic et al., (2014) reported that bilirubin, at a concentration of 0.5 mmol/L, disrupted the integrity of both axonal membranes and mitochondria within the axons. They noted that incubation in 0.5 mmol/L bilirubin for 7 hours had significantly enlarged mitochondria and vacuole-like bubbling as well as loss of definition of the cristae.

The golgi apparatus serves as a distribution system within the cells and acts as a controlling agent for movement of material both intracellular and extracellular. During the earlier stages of development of the arils (stages 1 and 2), more golgi bodies were noticed than at the later stages of development. At stage 5, no golgi bodies were observed. The same trend was observed with the vesicles. Vesicles mostly store, transport or digest cellular products and cellular by-products. There were more vesicles noted in the earlier stages than at the later stages. The presence of a large number of golgi bodies and vesicles in the early stages suggests high metabolic activity. As the cell matures, it becomes less energetic. Hence, the need for these organelles decreases (Robinson et al., 1999).

Lipids are omnipresent in plants, providing many important functions including storage of metabolic energy, protection against dehydration and pathogens and the absorption of light (Dyer et al., 2008). Lipids also contribute to the structure of membranes (Hall et al., 1974). In this study, it was observed that the amount of lipids increased as the arils mature. This probably serves as storage bodies as the seed matures. Also, as the cell begins to mature, the arils become much more structured. This structural characteristic may be attributed to these lipid molecules. Kauss, (1969) proposed that there is some evidence to implicate lipids as intermediates in polysaccharide biosynthesis in plants. Therefore, lipid molecules in the arils could aid in cell-wall polysaccharide formation, consequently forming more rigid walls as the cell matures.

The cytoplasm observed in the earlier stages (Stages 1 and 2) is somewhat less dense and as the aril matures the cytoplasm becomes granulated (Stage 3; Figure 14). In the later stages (Figure 15 and Figure 16), the cytoplasm becomes globular. This may be attributed to the presence of bilirubin. In addition, the cell thickenings at the junctions are suspected to be an accumulation of the bilirubin.

Conclusion

In this study, it was observed that the seed of *S. nicolai* undergoes five stages of maturation. As the seed matures, the seed colour as well as the size and texture changes. The arils found on the seeds undergo a similar maturation trend. The aril colour, texture, length and thickness also change as the cells mature. The aril colour changes from opaque to yellow and finally forming a deep orange colour in mature arils. This pigmentation could be attributed to the development of bilirubin in the cytoplasm and thus bilirubin could be the primary colour pigment in the arils as proposed by Pirone et al., (2009). The light and electron microscopic evaluation together with the Hall's and toluidine staining techniques provides visual confirmation of bilirubin's presence in the arils of *S. nicolai*. Furthermore, the trend of aril development in *S. nicolai* follows an analogous model to that of bilirubin metabolism in mammals.

Acknowledgments

This research was supported by the National Research Foundation (NRF) of South Africa. We would also like to thank Mr Vishal Bharuth and Mr Subashen Naidu from the Microscopy and Microanalysis Unit at UKZN, for their technical support and constant advice throughout this study.

References

1. Breimer, L. H., Wannamethee, G. and Ebrahim, S. (1995). Serum bilirubin and risk of ischemic heart disease in middle-aged British men. *Clin Chem* 41, 1504–1508.
2. Broderson, R. (1986) In bile pigments and jaundice. (Ostrow, JD ed) p158 Academic press, new York.
3. Carbajosa, V. J. and Carbonero, P. (2005). Seed maturation: developing an intrusive phase to accomplish a quiescent state. *Int. J. Dev. Biol*. 49: 645-651.
4. Chen, T. S. and Chen, P. S. (1984). Understanding the Liver. A History.Westport, CT: Greenwood Press.
5. Chowdhury, R. J., Wolkoff, A. W., Chowdhury, N. R., and Arias, I. M. (1995). The Metabolic Basis of Inherited Diseases, New York, McGraw-Hill.
6. Dyer, J. M., Stymne, S., Green, A. G. and Carlsson, A. S. (2008). High-value oils from plants. *Plant J*, 54, 640-655.
7. Frost, S. K. and Frost, P. G. H. (1981). Sunbird Pollination of *Strelitzia nicolai. Oecologia* 49:3, 379-384.
8. Gunning, B. E. S. and Steer, M. W. (1975). Ultrastructure and the biology of plant cells. Edward Arnold publishes, London.
9. Hall, M. J. (1960). A staining reaction for bilirubin in sections of tissues. *Amer. J. Clin. Path.*, 34:313-316.
10. Hall, J.L., Flowers, T. J. and Roberts, R. M. (1974). Plant and cell structure and metabolism. Longman, London.
11. Iłżecka, J. and Stelmasiak, Z. (2003). Serum bilirubin concentration in patients with amyotrophic lateral sclerosis. *Elsevier*. 8: 20-954.
12. Kauss, H. 1969. A plant mannosyl lipid acting in reversible transfer of mannose. *FEBS letter* 5, 81.
13. Lakovic, K., Ai, J., Abbondanza, J. D., Tariq, A., Sabri1, M., Alarfaj, A. K., Vasdev, P. and Macdonald, L. R. (2014). Bilirubin and its oxidation products damage brain white matter *Journal of Cerebral Blood Flow & Metabolism* 1–11.
14. Llesuy, S. F. and Tomaro, M. L. (1994). Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. *Biochimica et Biophysica Acta*. 1223: 9-14.
15. McDonagh, A. F. (2001). Turning green to gold. *Nat Struct Biol.* 200:98–200.
16. Pirone C. (2010). "Bilirubin: an Animal Pigment in the Zingiberales and Diverse Angiosperm Orders". FIU Electronic Theses and Dissertations.

17. Qian, T., Ma, R. H., Luo, X. B., Luo, Z. L., Zheng, M. P., and Yang, L. Q. (2013). A microstructural study of gallbladder stones using scanning electron microscopy. *Microscopy research and techniques.* 8:10:701.
18. Robinson, D. G., Baumer, M., Hinz, G. and Hohl, I. (1998). Vesicle transfer of storage proteins to the vacuole: the role of the Golgi apparatus and multivesicular bodies. *Journal of Plant Physiology* 152:650-667.
19. Watson, L., and Dallwitz, M. J. 1992. The families of flowering plants: descriptions, illustrations, identification, and information retrieval.
20. Zucker, S. D., Horn, P. S. and Serman, K. E. (2004). Serum bilirubin levels in the U.S. population: gender effect and inverse correlation with colorectal cancer. *Hepatology* 40, 827–835.

Chapter 7

Summary of study

7.1 General Discussion

According to the World Health Organisation (WHO), poverty and lack of modern medicine forces 65-80% of the world's population to depend entirely on plants for principle health care (WHO, 2008). While many conventional drugs or their precursors are derived from plants, there are fundamental differences between administering a pure chemical and the same chemical in a plant matrix. Synergy is an important concept in herbal pharmacology. In the context of chemical complexity, it applies if the action of a chemical mixture is greater than the arithmetical sum of the actions of the mixture's components i.e. the whole is greater than the sum of the individual parts (Mills and Bone, 2000).

The family *Strelitziaceae* consists of three genera, *Strelitzia*, *Phenakospermum* and *Ravenala*. *S. nicolai* similar to the other species of *Strelitzia*, produces black seeds with bright orange arils. The immature seeds of *S. nicolai* are edible. In addition, seeds of *S. nicolai* are used as a food source. The seeds are powdered, mixed with water and then baked as fritters over open fire. It is documentated that this is a complete meal but somewhat tasteless. There have been no traditional medicinal uses of *S. nicolai* to date (Van Wyk and Nigel, 2000). However, within the *Strelitzia* family, there have been a few reports of traditional therapeutic utilization. Cabanis et al., (1970) reported that the seed of *Ravenala madagascariensis* was cooked in milk and the arils containing fats benefited children. The ambakwaMthethwa tribe in KwaZulu-Natal traditionally used the strained concoctions from the flowers to treat swollen glands and venereal disease. In the Cape, the seeds were used to curdle milk (Van de Wall, 2011). In the nutrient starved tropical rainforest region, reports of the Amazonian forest dwellers food source consists of *Phenakospermum* (powdered seeds made into porridge). The arils are masticated and the juices consumed (Milton et al., 1991). There has to be some sort of protective medicinal benefit associated with these arils for the reason that the individuals in the forest regions that consume them are in good physical shape and they live extended lives in a nutrient difficient region. The recent discovery of bilirubin in *S. nicolai* by Pirone et al., (2009) has created a great deal of interest on the functional value of this compound in plants.

In the eukaryotic cell, the negative response inhibition of haem oxygenase (HO) activity by biliverdin is a methodology by which oxidation of haem is synchronized (Maines, 1992). Haem and iron homoeostasis in most eukaryotes is based on a balance flux between haem biosynthesis and haem oxygenase- mediated degradation. Biliverdin is the open tetrapyrrole creation of haem degradation by the haem oxygenase oxidation. In a variety of genera, including mammals and certain fish, biliverdin undergoes reduction to bilirubin (Kutty and Maines, 1984). In plants, biliverdin functions in a photoregulatory capacity and in cyanobacteria the alteration of biliverdin to bilirubin is important for normal phycobiliprotein synthesis (Terry and Lagarias, 1991). In animals bilirubin seems to have antioxidant and immunomodulatory property (Stocker et al., 1987) Hopkins (1996) reported that higher serum bilirubin levels are associated with decreased risk for early familial coronary artery disease. There are several studies that commonly demonstrate that low serum bilirubin concentrations are linked to an increased threat of certain pathological stipulations whereas placidly elevated serum bilirubin levels provide protection. On the basis of these findings this study was orchestrated. The primary task was to use the aril extract containing this bilirubin and to investigate it's *in vitro* and *in vivo* effects. Thereafter, aril ultra-structure was examined to gain knowledge of the structure of these arils and relate aril structure to function.

It has been established that oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and many more (Kamuhabwa et al., 2000). In carcinogenesis, reactive oxygen species are responsible for initiating the multistage carcinogenetic process starting with DNA damage and accumulation of genetic events in one or few cell lines which lead to progressively dysplastic cellular appearance, deregulated cell growth and finally carcinoma (Van Cruchten and Van Den Broeck, 2002). Therefore, a good antioxidant will also be a good anti-cancer agent.

In this particular study the aril extract containing bilirubin was tested for its antioxidant (DPPH) and anti-cancer (XTT) activity *in vitro*. A standard synthetic bilirubin was also tested for its activity. The DPPH activity showed that the aril extract acts as a more potent antioxidant than bilirubin on its own. The XTT assay also gave similar results. The anti-cancer results were further validated by the Annexin V assay whereby apoptosis was quantified and the acridine orange-ethidium bromide staining was used to detect morphological changes of the Hela cells. This assessment of bilirubin found in the arils and the standard showed the extract and standard both work in a similar manner to the positive control Taxol®. Nevertheless, the aril extract is much more powerful than bilirubin on its own against Hela cell lines. These results clearly demonstrate that bilirubin works synergistically as a

powerful antioxidant and anti-cancer agent with other compounds found in the arils than the pure bilirubin.

The *in vitro* results showed the effectiveness of the aril extract as an antioxidant and anti-cancer agent. Though the indigenous Americans from the Amazonian forest consume the seeds and aril of *P. guyannense*, toxicity testing *in vivo* is vital in the selection of newly formulated drugs before it can be used on humans. This will allow for the authentication of the substance if the substance produces possible danger and the characterization of the nature of the side effects. In this particular study, Sprague Dawley rats were used to test the acute toxicity of the extract at 250µg/ml. This concentration was the optimum for the anti-cancer study and, hence, used as a basis for the animal trials. In regular metabolism, if bilirubin is not excreted the accumulation (at high levels) leads to toxicity and neurological damage. The initial toxicity can be seen on the skin and the liver. Pathological and histological results from this acute study showed that there was a degree of kidney damage that did take place. However, it cannot be concluded that administering the aril extract resulted in this effect since this effect was also found in the control groups. Often in animal units, the feed is contaminated easily which results in false positive results. This study indicates that the aril extract could be used as a therapeutic intervention. However, the correct dose needs to be established. There is a fine line between the toxic doses versus the therapeutic dose of bilirubin. The paramount importance is definitely to establish this range. Moreover this acute primary study served as a basis for aril intervention. However, a chronic more comprehensive and exhaustive study still needs to be commenced to verify the safety of the aril extract containing bilirubin.

The next sector of this study focused on developing an understanding of the structure of the arils. Firstly, based on aril colour, size and thickness the arils were categorised in five different stages of maturation. An attempt was made to view the surface structures of the arils at the different stages. The initial attempt was the normal conventional SEM method. However, the challenge lay in the fact that the arils in the earlier stages were filled with mucilage, so when certain preparation procedures were carried out on the arils, the arils became distorted. Owing to the delicate nature of the arils, certain preparation methods introduced artefacts. In this particular study, six different methods were tested. Those methods include: (a) fresh and uncoated samples viewed under a partial vacuum SEM (VPSEM), (b) cryo-fixation of fresh material, followed by freeze drying for 48 hours, (c) cryo-fixation of fresh material followed by freeze drying for 24 hours, (d) chemical fixation using a solution of formalin: acetic acid alcohol (FAA), (e) conventional chemical fixation of material using glutaraldehyde (GA) and (f) cryo-fixation of fresh material, followed by sputter coating and viewing under a high vacuum on a cold stage. Preparation methodologies were assessed based on the quality and reliability of the resulting illustrations, as observed in the images taken. From this

experimentation it can be deduced that cryo-SEM works unsurpassed for the preparation and viewing the surface morphology of the arils. This method introduced the least amount of artefacts to the sample. It also allowed the sample to be preserved close to its natural state without a great deal of distortion. Stokes, (2008) mentioned that every specimen and its imaging history will be different and many of the operating parameters are interdependent as well as specimen-dependent. This became evidently conscious in this study.

The final trial of this study intended to interlink the structure and function of the aril cells as revealed by different techniques other than SEM. In this chapter, stereomicroscopical and light microscopical analysis of the arils was studied. This included the examination of stained and unstained arils at the different stages of maturation. Toluidine blue was used to stain the arils in cross and longitudinal sections in order to see contrast in the arils embedded in resin. The modified Hall's staining was used to locate the specific surface areas that appeared to be bilirubin deposits. The TEM and cryo-SEM were utilized in order to identify internal and external structures at high magnifications. The findings of this study correlate to Swanson's (1985) idea that "every science has one or more periods of time when it burst, its bounds and exhibit an extraordinary pattern of growth". Taking into consideration all the microscopical analyses (light, staining and electron microscopy), there was a similar developmental trend that was observed in the five stages of aril maturation. The arils are more or less translucent at stage one, and move from white at stage two to yellow at stage three, orange at stage four and deep orange at stage five. Bilirubin, as proposed by Pirone et al., (2009), is suspected to be the main colour causing pigment in the arils. The toluidine blue, TEM and cryo-SEM shows that as the arils develop from stage one to stage five, there is an increase in the pigment internally that is thought to be bilirubin. The TEM images suggest that, as the aril matures it seems to follow a similar trend to that of xylem maturation whereby in the initial stages there are masses of organelles present (vesicles, golgi bodies and endoplasmic reticula), which indicates metabolically active cells. Conversely, as the aril matures the pigment is crammed in the cell with a lack of these vigorous bodies. The cryo-SEM images indicated that the structural components of the arils from stage three onwards are composed of radial clump like structures similar to that of bilirubinate particles found in pigment stones. The modified Hall's staining further verifies this hypothesis. As the stages increase the surface occurrence of bilirubin droplets also increases. Gunning and Steer, (1975) advocated that biological structures are better interpreted on an evolutionary basis rather than assuming its design, in other words by considering that they have been shaped in the course of evolution by natural selection and survival of those types which are in greatest harmony with the conditions under which they exist.

7.2 Conclusion

Subsequent to scientific verification of the presence of bilirubin using TLC and NMR, this study demonstrated the application of the use of arils containing bilirubin of *S. nicolai* against free radicals and Hela cells. This confirmed the action of bilirubin as a powerful antioxidant in the body. The acute toxicity assays could not guarantee the safety of this extract. Nonetheless, more elaborate chronic assays are needed to give concrete evidence of its safety.

This study also pointed out the difficulty in treating aril tissue for SEM. This study has concluded that different biological material needs to be prepared differently according to their constituents for SEM.

Light and electron microscopy, together with the modified Hall's staining, showed the presence of bilirubin on the surface of the arils. TEM and SEM illustrated the ultrastructure of the five different stages of aril development. It is concluded in this study that bilirubin's occurrence in the cell increases and accumulates as the aril matures. This could be an explanation of the colour changes of the aril from opaque to yellow to finally a deep orange when fully mature.

The discovery of the only human compound in a plant has set in motion an array of questions regarding bilirubin's existence in the human body and in the plant. This study provides the basis of insight of what one may consider to be a bizarre and astonishing leap forward in the field of Medical Science. I remain optimistic that the arils of *S. nicolai* containing bilirubin will, in the near future, play an integral role in traditional homeopathic and allopathic medicine, not only in South Africa but in the world.

7.3 Recommendations for future research

The basis of cancer methodologies developed on Hela cells in this study can be used to further assess the apoptotic activity and cytotoxicity of the aril extract against other cancer cell lines. Further research is required to determine the effectiveness of the aril extract in treating cancers. This future study should also include the extract effects on non-cancerous cell lines. Bilirubin should also be isolated from the extract and activity should be compared with the extract as a whole and the synthetic bilirubin.

In order to confirm the safety of the extract, an acute study animal trial was undertaken. However, the result of this study was inadequate to conclude the safety of the extract if administered intravenously. A longer, prolonged chronic trial needs to be undertaken. If the safety of the extract is justified in this particular trial, then given that bilirubin is thought to be a powerful antioxidant, the antioxidant potential should be tested *in vivo.* This study can also investigate the anti-diabetic and anti-hypertensive potential of this extract, the isolated bilirubin and the synthetic bilirubin.

This particular study formed the basis as to what appears to be bilirubin in the arils of *S. nicolai* using light and electron microscopy. To further validate the presence of bilirubin at each stage of aril maturation, a highly specific technique of gold tagging may perhaps be used. This technique encompasses the use of colloidal gold particles which are attached to a secondary antibody which is sequentially attached to a primary antibody produced to bind explicitly to an antigen. Due to the specificity of this TEM technique, the presence of bilirubin may be accurately pin-pointed at different stages of aril development.

Phytomedicines still play an important role in meeting basic health care of local communities in South Africa and in the undeveloped parts of the world. Potential prospects of the arils containing bilirubin, in the medical sector as applications in the pharmaceutical, nutraceutical cosmetic industries and more importantly the management of human diseases could be exceptionally promising.

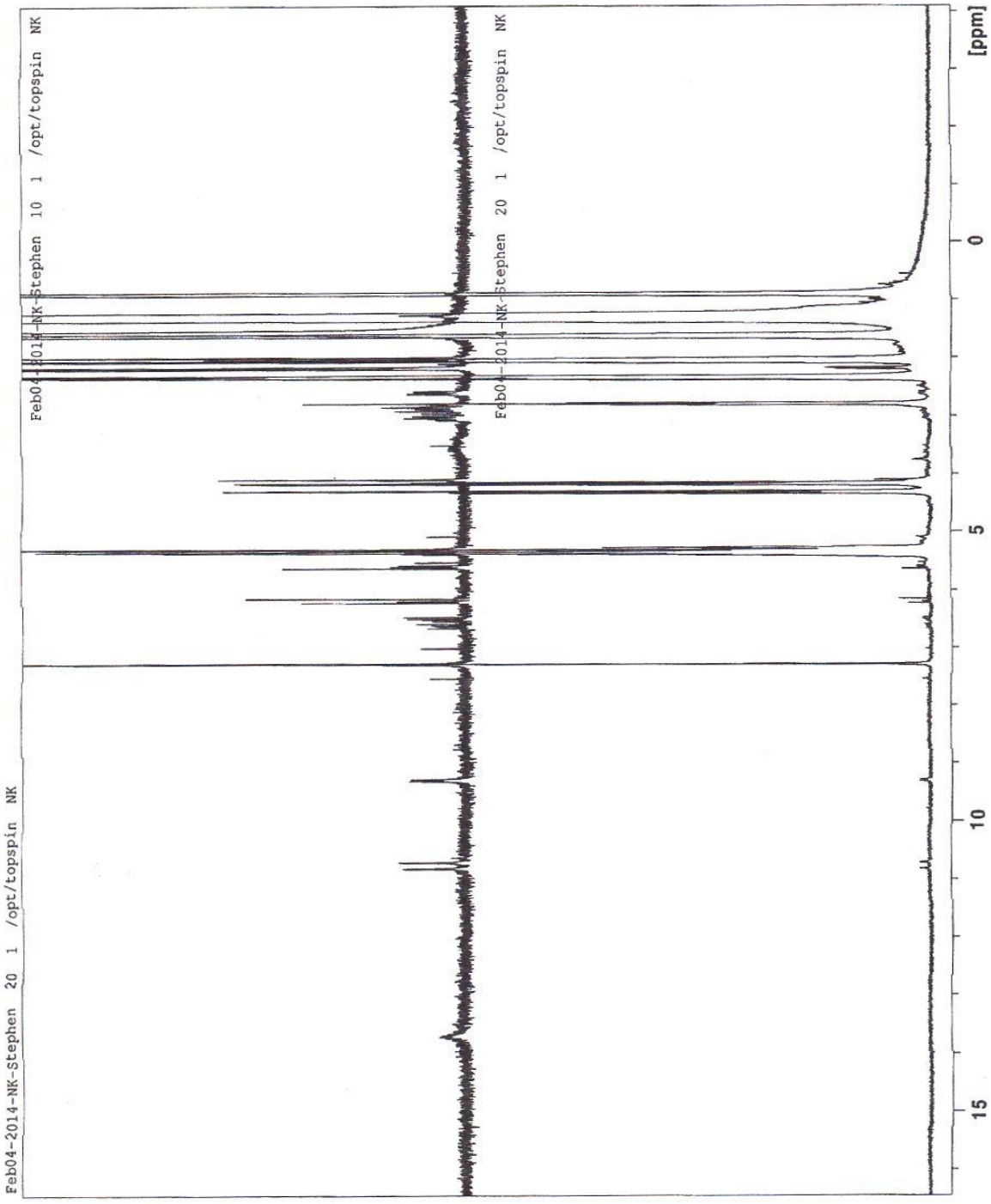
References

1. Cabanis, Y., Chabu, L. and Chabu, F. 1970. Plants and plant communities of Madagascar and the Mascarene. *Bureau for Development of Agricultural Production*, Tananarive.
2. Hopkins, P.N., Wu, L.L., Hunt, S.C., James, B.C., Vincent, M.G. and Williams, R.R. 1996. Higher serum bilirubin is associated with decreased risk for early familial coronary artery disease. *Arterioscler Thromb Vasc Biol.*16:2: 250-5.
3. Kamuhabwa, A., Nshimo, C., and Witte D. 2000. Cytotoxicity of some medicinal plant extracts used in Tanzanian traditional medicine. *Journal of Ethnopharmacology* 70: 143-149.
4. Kutty, R.K., and Maines, M.D. 1984. Hepatic heme metabolism: possible role of biliverdin in the regulation of heme oxygenase activity. *Biochem Biophys Res Commun.* 18:122:1:40-6.
5. Maines, M.D. 1999. Heme Oxygenase: Clinical Applications and Functions. CRC Press, Inc., Boca Raton, Florida.
6. Mills, S. and Bone, K. 2000. Principles and practice of phytotheraphy. Churchhill Livingstone, United states of America.
7. Milton, K., Knight, C.D. and Crowe, I. 1991. Comparative aspects of diet of Amazonian forest dwellers. *Phil. Trans*. 334: 253-263.
8. Pirone, C., Quirke, M.J., Priestap, H.A. and Lee, D.W. 2009. The Animal Pigment Bilirubin Discovered in Plants. *J Am Chem Soc.* 131:8: 2830.
9. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A.N., and Ames, B.N. 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* 235: 1043–1048.
10. Stokes, D.J. 2008. Principles and practice of variable pressure/environmental scanning electron microscopy. John Wiley and Sons, United Kingdom.
11. Swanson, C.P. 1985. The cell. Prentice-Hall International, INC, London.
12. Terry, M.J. and Lagarias, J.C. 1991. Holophytochrome assembly. Coupled assay for phytochromobilin synthase in organelles. *J. Biol. Chem.* 266: 22215–22221.
13. Van Cruchten, S. and Van Den Broeck, W. 2002. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anat Histol Embryol* 31: 214-223.
14. Van Wyk, B.E., Nigel, G. 2000. People's plants. A guide to useful plants of Southern Africa. Briza Publications, South Africa.
15. Winter, J and Xaba, P. 2011. *Strelitzia juncea*. http://www.plantzafrica.com/plantqrs/strelitzjun.htm.

16. World Health Organization (2008). Regional office for western Pacific, research guidlines for evaluating the safety and efficacy of herbal medicines, Manila.

Appendix 1

NMR spectroscopy of bilirubin standard and *S. nicolai* aril extract



Appendix 2

Animal ethics and histology of treated animals

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

18 January 2016

Ms Depika Dwarka
School of Life Sciences
Westville Campus

Dear Ms Dwarka,

Protocol reference number: 075/14/Animal
Project title: Structural, chemical and physiological properties of bilirubin isolated from arils of *Strelitzia nicolai*

Full Approval – Renewal Application

With regards to your application received on 08 January 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue.

I take this opportunity of wishing you everything of the best with your study.

You[redacted]

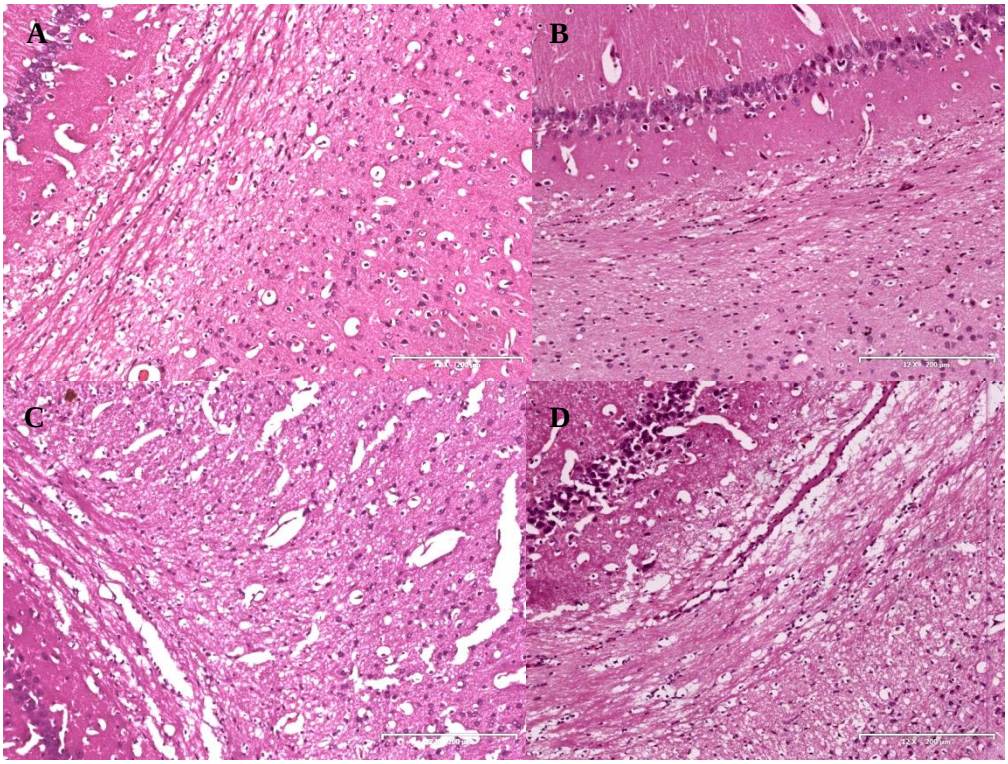
.....

Prof
Chair: Animal Research Ethics Committee

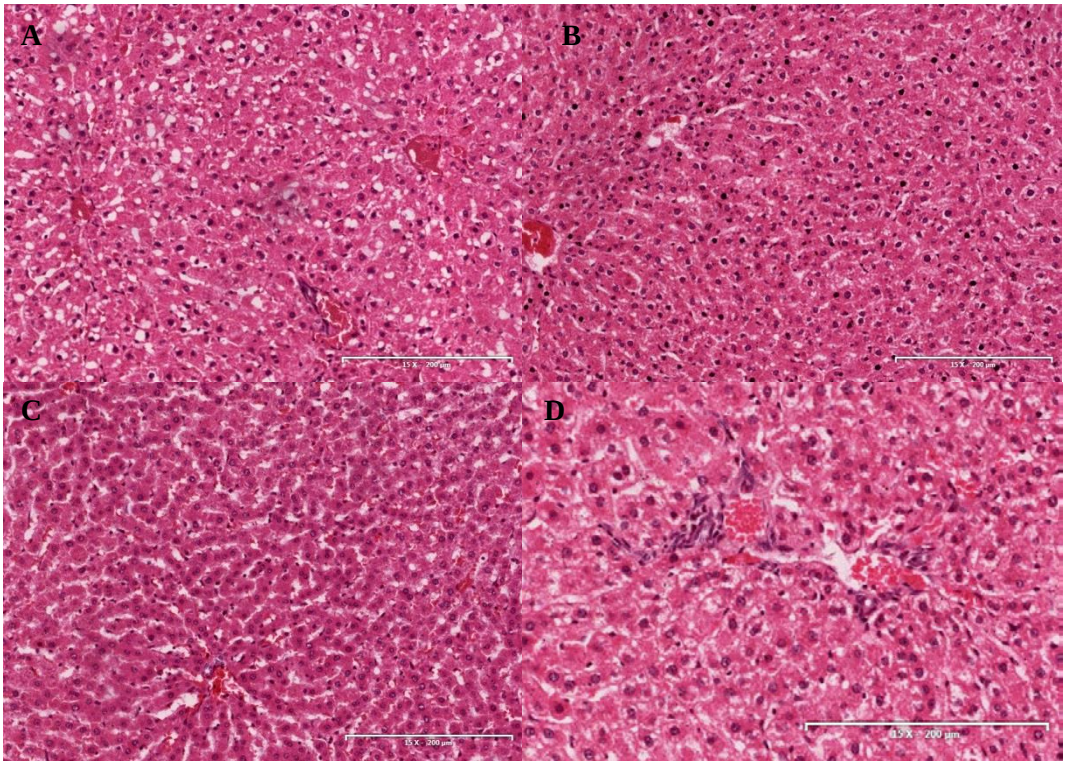
/ms

Cc Supervisor: H Baijnath
Cc Dean & Head of School: Dr Samson Mukaratirwa
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light

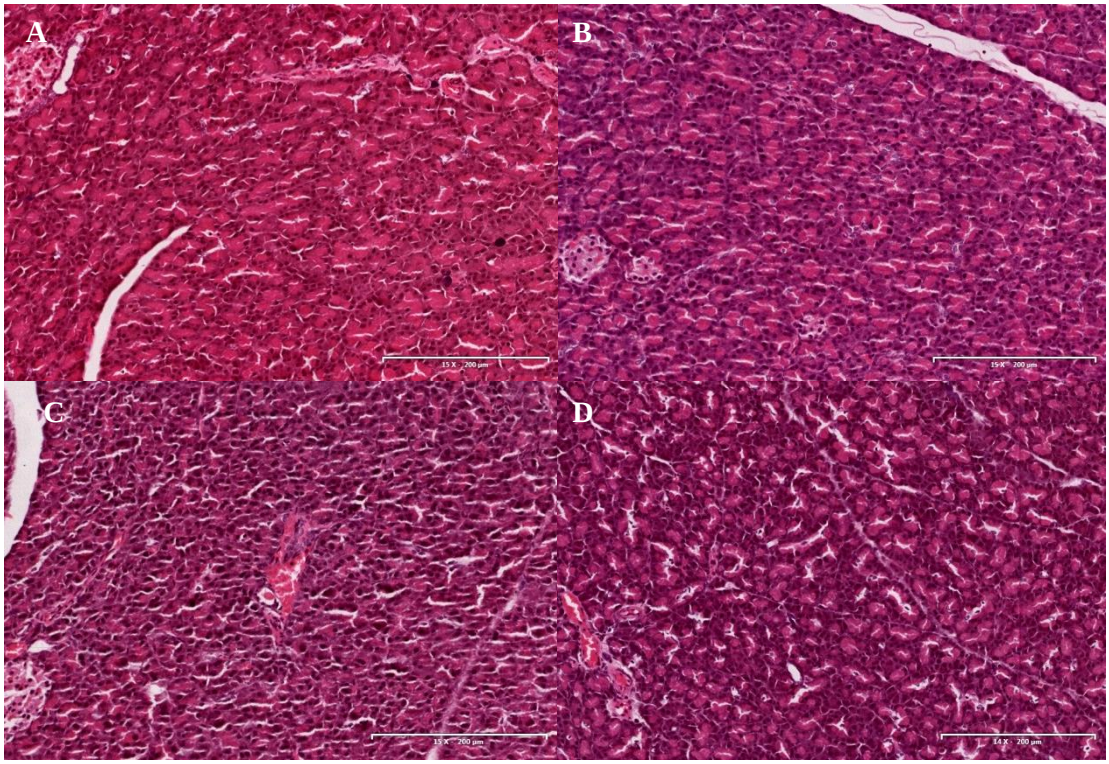
Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx



Brain: (A) control, (B) 24 hours, (C) 48 hours and (D) 72 hours



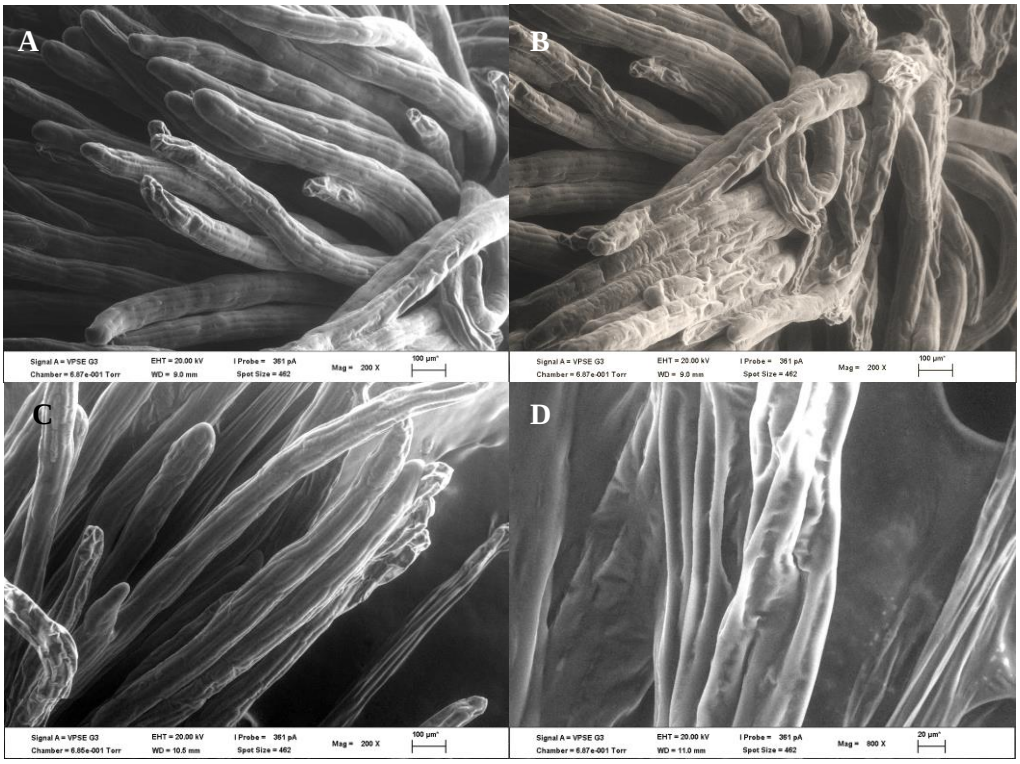
Liver: (A) control, (B) 24 hours, (C) 48 hours and (D) 72 hours



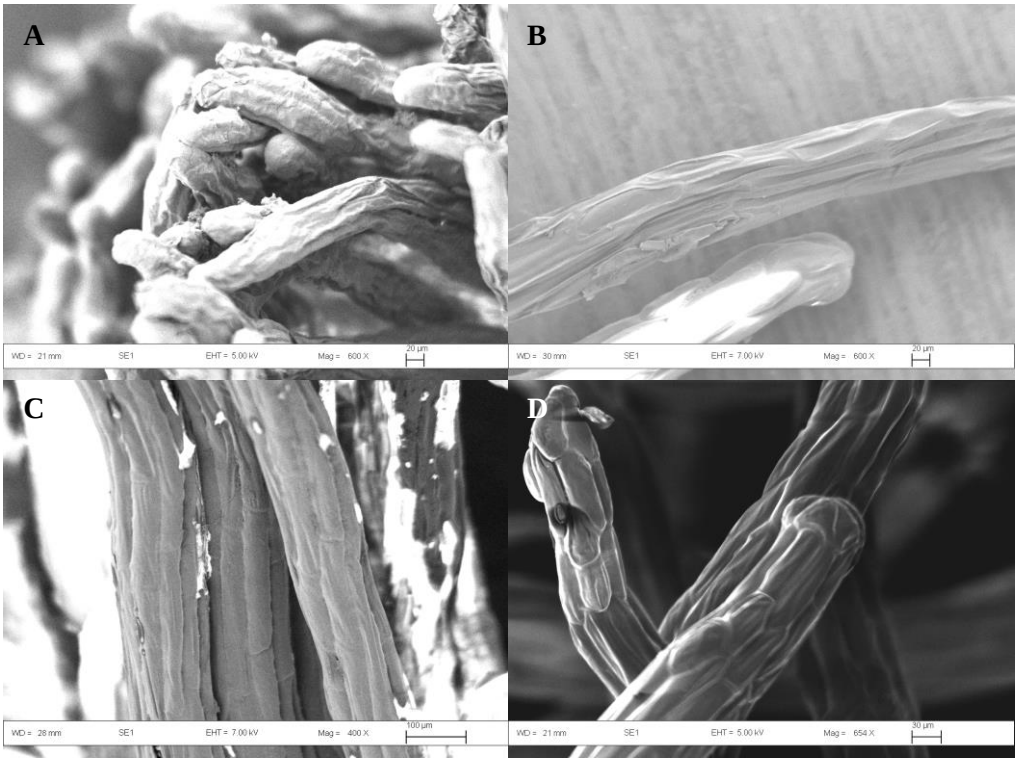
Pancreas: (A) control, (B) 24 hours, (C) 48 hours and (D) 72 hours

Appendix 3

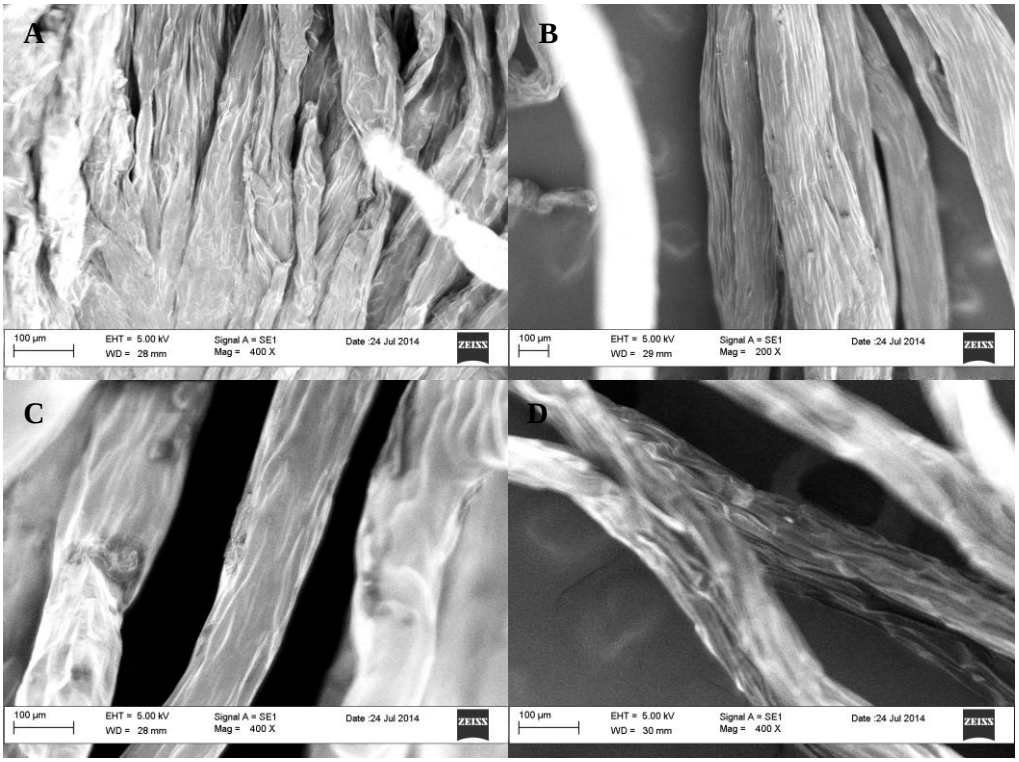
SEM micrographs of stages 2, 3, 4 and 5 using different preparation methodologies



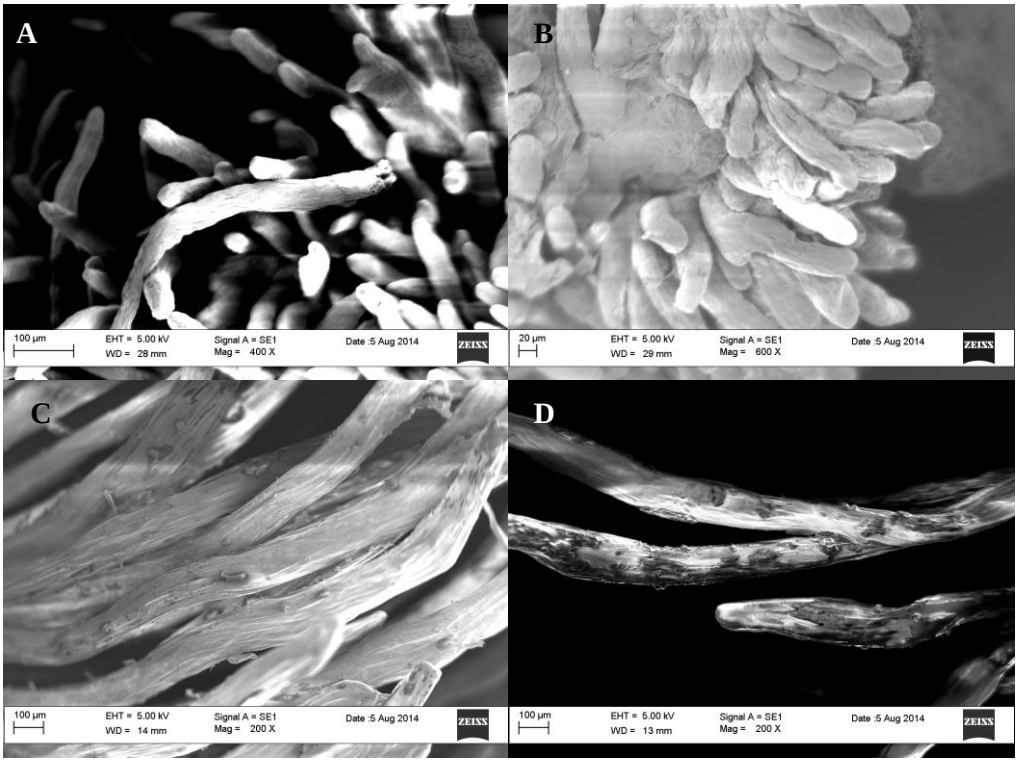
VP SEM: stages (A) 2, (B) 3, (C) 4 and (D) 5.



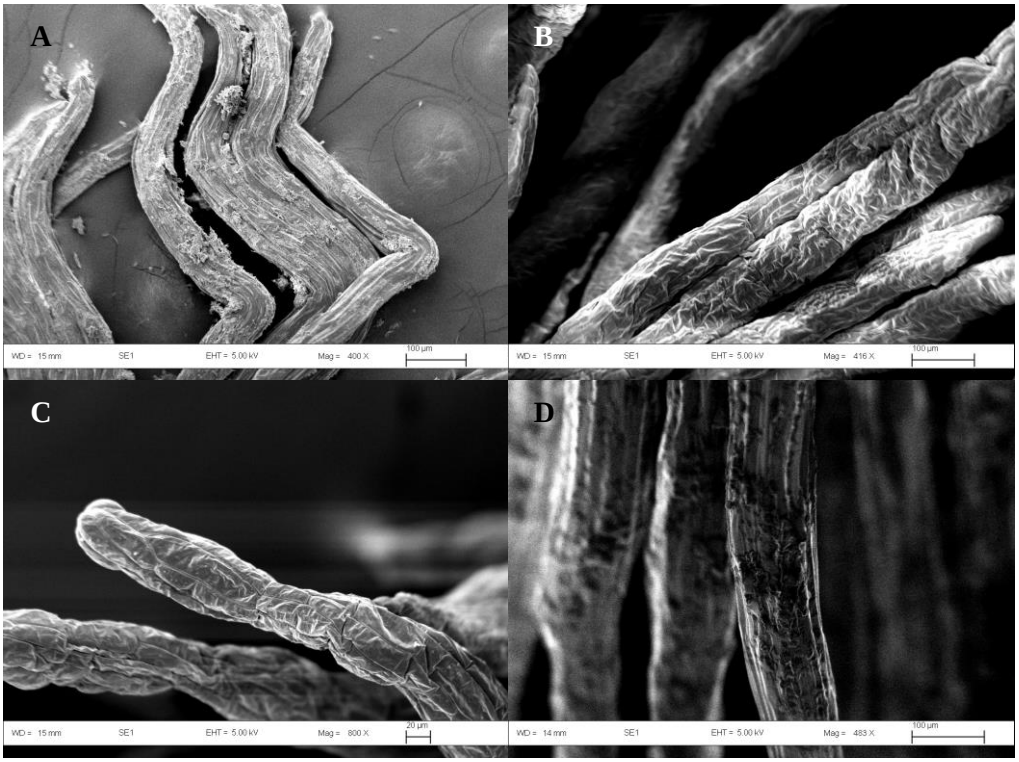
Freeze dried 48 hours: stages (A) 2, (B) 3, (C) 4 and (D) 5



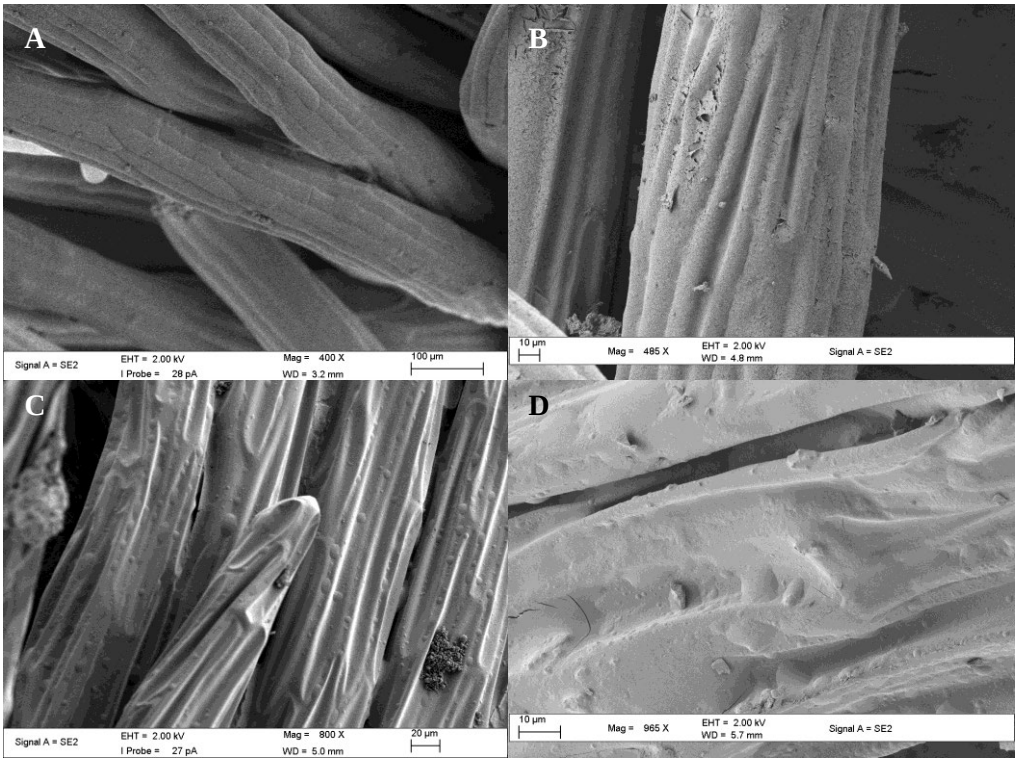
Freeze dried 24 hours: stages (A) 2, (B) 3, (C) 4 and (D) 5



FAA: stages (A) 2, (B) 3, (C) 4 and (D) 5



Conventional glutaraldehyde: stages (A) 2, (B) 3, (C) 4 and (D) 5



Cryo-SEM: stages (A) 2, (B) 3, (C) 4 and (D) 5