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PTEROCARPUS ANGOLENSIS CRUDE EXTRACTS INDUCE THE EXPRESSION OF COLLAGEN TYPE II IN ARTICULAR CARTILAGE *IN VITRO*

Cornelius Cano Ssemakalu [1], Mapula Razwinani [1], Makwese Johannes Maepa [1] and Keolebogile Shirley Motaung [1#]

[1]Department of Biomedical Sciences, Faculty of Science, Tshwane University of Technology, Pretoria 0002, South Africa

***corresponding author E-mail:** motaungsckm@tut.ac.za

Abstract

Background: *Pterocarpus angolensis* (*P. angolensis*) is a large deciduous tree native to Sub-Saharan Africa. This tree has traditionally been used to treat malaria, gonorrhoea, inflammation and wounds. The use of *P. angolensis* for tissue engineering has not been explored. This study investigated the potential of the bark and root water extracts of *P. angolensis* to induce the expression of collagen type II protein in the articular chondrocytes.
Materials and Methods: Surface and middle zone chondrocytes, as well as explants, were stimulated with crude root and bark plant extracts at different concentrations. After 96 hours of stimulation, microscopic observation, XCELLigence assay and collagen type II ELISA, histology and immunohistochemistry assays were done.
Results and Conclusion: Results showed no significant difference in the cell index between the controls and chondrocytes that had been treated with the plant extracts at concentrations of 15 and 30 µg/ml. A significant increase in the expression of collagen type II protein by the chondrocytes was observed and found to be optimal at a concentration of 30 µg/ml. There was an increase in the production of proteoglycans. However, the plant extracts at a concentration of 50 µg/ml induced apoptosis in the middle zone chondrocytes. These results show the potential of *P. angolensis* extracts in chondrocyte tissue regeneration. This potential could be exploited in the treatment of osteoarthritis.

Keywords: *P. angolensis*, medical plants, chondrocytes, collagen type II, arthritis

Introduction

Pterocarpus angolensis (P. angolensis), also known as moroto (Sotho) or umbilo (Zulu), is a large deciduous tree native to the Sub-Saharan African region (Cameron et al., 2009; Geldenhuys, 2013; van Wyk & van Wyk, 2013).

This tree usually grows to about 16 meters tall, has a dark brown bark and a high, wide, crowned canopy of shiny compound leaves. Wood from *P. angolensis* has been used to make furniture because of its resistance to termites and borer (Geldenhuys, 2013). Besides furniture, this tree has traditionally been used to treat diseases and medical conditions such as malaria, gonorrhea, ringworm, skin inflammation, wounds, stomach ache and poor breast milk supply (Geldenhuys, 2013; van Wyk & van Wyk, 2013).

The use of *P. angolensis* to heal wounds suggests that this tree contains compounds that stimulate the regeneration of torn tissue. The regeneration of torn tissue in humans has been linked to an increase in the expression of compounds such as hyaluronan, chondroitin sulphate, aggrecan and collagen (Fraser et al., 2003; Greco et al., 1998; Ko et al., 2009; Wood, 1960).

Collagen is the main structural protein of various connective tissues in animals. This protein is produced by cell types derived from mesenchyme such as fibroblasts, osteoblasts, chondroblasts and smooth muscle cells. Chondrocytes are responsible for the production of collagen found within the cartilage matrix. Collagen type II in particular is the principal component of articular cartilage and has various benefits. However, these benefits are reduced in patients suffering from arthritis owing to the degradation of the cartilage protein by collagenase (Fraser et al., 2003).
The degradation of cartilage material results in osteoarthritis (OA) and rheumatoid arthritis (RA). The number of individuals suffering from osteoarthritis is on the rise worldwide (Johnson & Hunter, 2014; Zhang & Jordan, 2010).

This condition is more prevalent in women than in men (Akinpelu et al., 2011; Johnson & Hunter, 2014; Zhang & Jordan, 2010). On the other hand, RA has been reported to be one of the main causes of work disability (Butcher, 2008). The rise in OA and RA has a direct impact on health care and public health systems.

Both OA and RA have been remedied through the use of glucosamine and chondroitin supplements, undenatured collagen type II (Crowley et al., 2009; Trentham et al., 1993) and herbal medicines (Cameron et al., 2009; Ernst, 2006; Long et al., 2001), to mention but a few. In Africa, plant extracts such as those from *Ricinus communis* (castor oil) or *Harpagophytum procumbens* (devil's claw) (Cameron et al., 2009) have been used to alleviate symptoms associated with OA or RA. *P. angolensis*, because of its healing properties mentioned earlier, may have the ability to remedy the symptoms of arthritis. This study investigated the ability of the bark and root water extracts of *P. angolensis* to induce the expression of collagen type II protein by articular chondrocytes *in vitro*.

Materials and Methods

Plant collection

The roots and bark of *P. angolensis* were collected from Venda, Limpopo Province of South Africa in September 2014. Identification was done by a botanist at the University of Venda, where voucher specimens were deposited.

Preparation of the bark and root crude extracts

The bark and roots were washed with water to remove excess soil and then air-dried at room temperature for two weeks. Thereafter the plant material was ground in a Wiley mill grinder and soaked in water with intermittent shaking for 24 hours. The extracts were then filtered, the filtrate was frozen at -70°C and thereafter lyophilized for three to four days. The lyophilized plant material was stored at -70°C until it was required for biological assays.

Tissue acquisition and cell culture

Stifles from three-month-old pigs were obtained from an abattoir and dissected under aseptic conditions to expose the femoral condyles, as previously described (Khalafi et al., 2007; Niikura & Reddi, 2007). The superficial and middle zone cartilage of the femoral condyles were harvested using a dermatome (Integra, Plainsboro, NJ, USA) and digested with 0.2% collagenase-P (Roche Pharmaceuticals, Nutley, NJ, USA) for three hours to release the chondrocytes. The chondrocytes were then plated as monolayers at a density of 1×10^5 cells/well in DMEM/F-12 medium containing 1% fetal bovine serum and incubated at 37°C in a 5% Carbon dioxide atmosphere.

Stimulation of the chondrocytes with crude bark and root water extracts

After 24 hours of incubation, the chondrocytes were washed, resuspended in serum-free DMEM/F-12 medium supplemented with insulin transferrin selenium (ITS) + Premix (BD Bioscience, Bedford, MA, USA) and seeded at a concentration of 1 x 10^5 cells/ml. They were then stimulated with the bark and root extracts from *P. angolensis* at concentrations of 15, 30 and 50 µg/ml and hydrogen peroxide (2 µg/ml) for 96 hours. Hydrogen peroxide and the untreated chondrocytes were used as controls.

Cell viability assay

The plant extracts were assessed for their effects on the viability of the chondrocyte using the XCELLigence assays (RTCA DP Instrument). In brief, 100 µl of the serum-free culture medium containing 1 x 10^5 cells/ml was transferred into each well of the E-plate 16 (ACEA Biosciences,Inc).The cells were then allowed to adhere to the plate surface at 37ºC in a 5% CO_2 atmosphere. After 24 hours of incubation, the cells were treated with bark and root plant extracts and H_2O_2 at the concentration described previously. Unstimulated chondrocytes from both the superficial and middle zone were used as biological controls. After 96 hours of incubation the cells were assessed for their viability, based on the cell index.

Assessment of the cell morphology

After 96 hours of stimulation, the morphology of the chondrocytes was assessed using an inverted light microscope (Leica DMRB, Weitzl, Germany).

Apoptosis analysis

The ability of the plant extracts (50 µg/ml) to induce apoptosis was investigated using the annexin V-FITC kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, 1 x 10^6 cells were stained with annexin V conjugated with FITC. After 15 minutes of incubation the unbound annexin V-FITC was washed off and the cells where counter-stained with propidium iodide (100 µg/ml) and immediately analyzed with a macs quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

Collagen type II enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) kit was used for quantitative determination of collagen type II (MD Biosciences, St. Paul, MN, USA) protein levels in the conditioned culture media according to the manufacturer's instructions. The assay is based on the competitive inhibition of primary antibody binding to collagen type II-coated plates

Histological and immunohistochemical analysis

After 96 hours of incubation with crude root water extract at 15 µg/ml the articular cartilage tissue explants were fixed in 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis MO) for 24 hours at room temperature. The cartilage tissue explants were then vertically sectioned from the surface to the bottom into 5 µm thick tissue sections. These tissue sections were then deparaffinized in xylene and rehydrated in an alcohol gradient. The tissue sections were then stained with hematoxylin and eosin (H&E) and safranin-O. The tissue sections were also assessed for the presence of collagen type II using the novalink min polymer detection system kit (Leica Microsystems, Newcastle, UK) according to the instruction

manual. In brief, the tissue sections were flooded with 1% hydrogen peroxidase for 30 minutes at room temperature so as to block endogenous peroxidase activity. The hydrogen peroxidase was washed off three times with phosphate buffered saline (PBS). Then rabbit polyclonal collagen type II antibody at a dilution factor of 1:1000 was added to the sections, which were then incubated at 4ºC overnight. Following the overnight incubation the sections were washed three times with PBS and incubated with a biotinylated antibody at room temperature. After 1 hour of incubation, the sections were stained with diaminobenzidine, which was shortly thereafter washed off, and then counter-stained with Mayer's hematoxylin. The sections were air-dried and then mounted with entallin mounting medium before the microscopic examination. To test for false binding of the secondary antibody, control experiments lacking the primary antibody were done in parallel.

Statistical analysis

The measure of the significance of the difference observed between treatments was determined using the Student's t-test; $p<0.05$ was considered statistically significant.

Results

Cell viability

The viability of the chondrocytes was continuously monitored by XCELLigence and expressed as a slope. In this study the slope represented the rate of change of the cell index for the entire duration of incubation. Results showed that the crude bark extract at concentrations of 15 and 30 µg/ml induced a greater increase in the cell index of the superficial zone chondrocytes than that observed in similar but unstimulated chondrocytes (Figure 1). When the crude bark extract was used to stimulate middle zone chondrocytes at concentrations of 15 and 30 µg/ml, there was no significant change in cell index when compared to the unstimulated chondrocytes. Both the superficial and middle zone chondrocytes exhibited a decrease in cell index when stimulated with the bark extract at a concentration of 50 µg/ml (Figure 1). The observed decrease in cell index of the middle zone chondrocytes was significant at a $p < 0.05$ level when compared to the unstimulated chondrocytes. The crude root extract slightly reduced the cell index of the superficial zone chondrocytes; however, this reduction was not significant when compared to that observed in the unstimulated chondrocytes. A decrease in cell index of the middle zone chondrocytes was also observed following their stimulation with the root extract. The observed decrease in the cell index was not significant in comparison to the unstimulated chondrocytes, with the exception of the cells stimulated with the root extract at a concentration of 50 µg/ml.

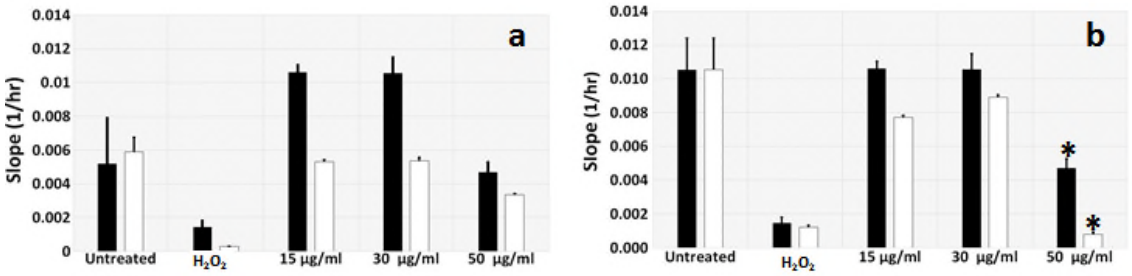

Figure 1: Viability assay for superficial (a) and middle (b) zone chondrocytes stimulated with crude bark (black bars) and root (white bars) extracts from *P. angolensis* at different concentrations. Untreated and H_2O_2-treated chondrocytes were used as controls. Error bars indicate the standard error of means from duplicate experiments. * indicates means that had a $p < 0.05$ in comparison with the untreated chondrocytes.

***P. angolensis* crude water extracts did not alter the cellular morphology of the chondrocytes**

The morphology of chondrocytes stimulated with different concentrations of the crude bark and root water extracts of *P. angolensis* as well as H_2O_2 was assessed under a bright field microscope. The untreated chondrocytes were used as comparison controls. After 96 hours of stimulation, cells with an elliptical/spheroidal shape attached to the bottom of the tissue culture flasks were observed in treatments involving both the bark and root crude water extracts from *P. angolensis*. Cells with a similar morphology were observed in the untreated samples. The cells in samples treated with H_2O_2 were neither elliptical in shape nor attached to the bottom of the tissue culture flask (Figure 2). No morphological difference was observed between the cells that were treated with either the crude bark or root water extract. Furthermore, there were no observable differences in the morphology between the chondrocytes extracted from the superficial and middle zones (Figure 2.

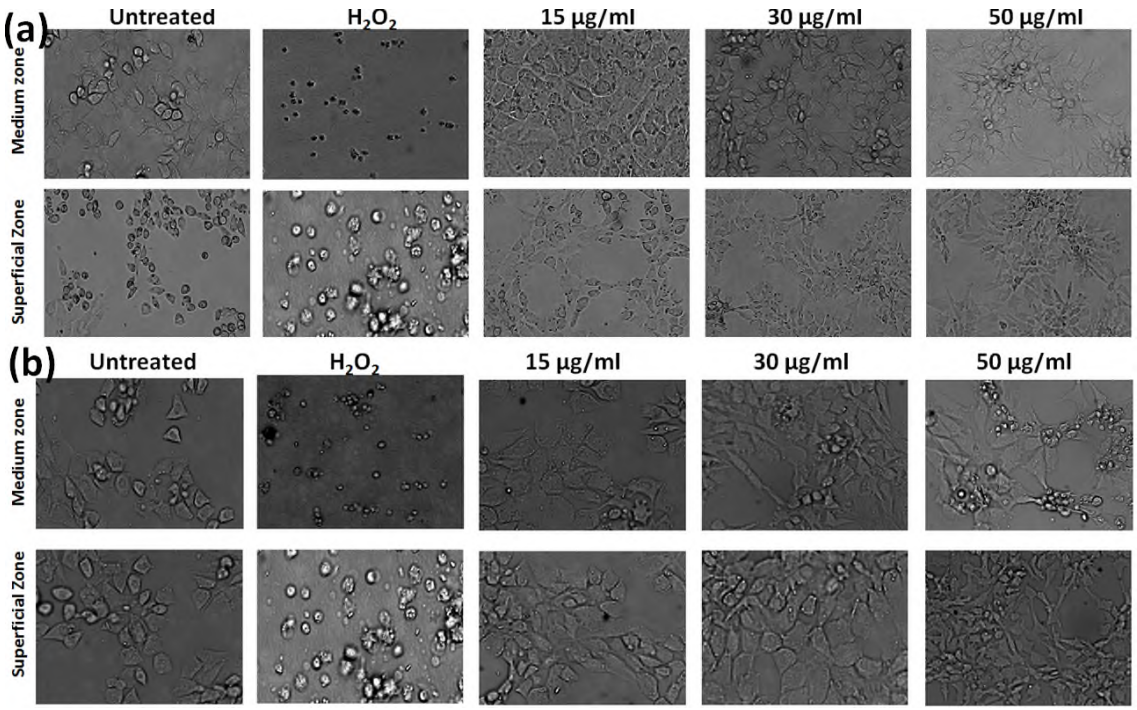

Figure 2: Morphology of surface and middle zone chondrocytes stimulated with crude bark extract (a) and crude water extract (b) from *P. angolensis* at different concentrations. The chondrocytes were stimulated with crude plant water extracts for a period of 96 hours. Untreated and H_2O_2-treated chondrocytes were used as comparison controls. Magnification X40.

***P. angolensis* crude water extracts at 50 µg/ml induced apoptosis in middle zone chondrocytes**

P. angolensis crude bark and root extracts at a concentration of 50 µg/ml were shown to reduce the cell index significantly. It was therefore important to establish if the observed reduction in cell index was as a result of apoptosis. Both the superficial and middle zone chondrocytes were stimulated with the crude bark and root water extracts from *P. angolensis* for a period of 96 hours. The unstimulated chondrocytes as well as those stimulated with hydrogen peroxide were used as comparison controls. The results showed that the majority of the cell population was viable. On the other hand, there were a few cells that displayed annexin V on their cell surface. The amount of annexin V observed within the middle zone chondrocytes stimulated with either the crude bark or root water extracts was greater than that observed in the unstimulated chondrocytes. Although small, the difference in the percentage of cells undergoing apoptosis between the chondrocytes that were treated with either the crude bark or root extract and those that were unstimulated was significant at a $p<0.05$ level (Figure 3). More than 90% of the superficial and middle zone chondrocytes treated with hydrogen peroxide had annexin V on their cell surface (Figure 3).

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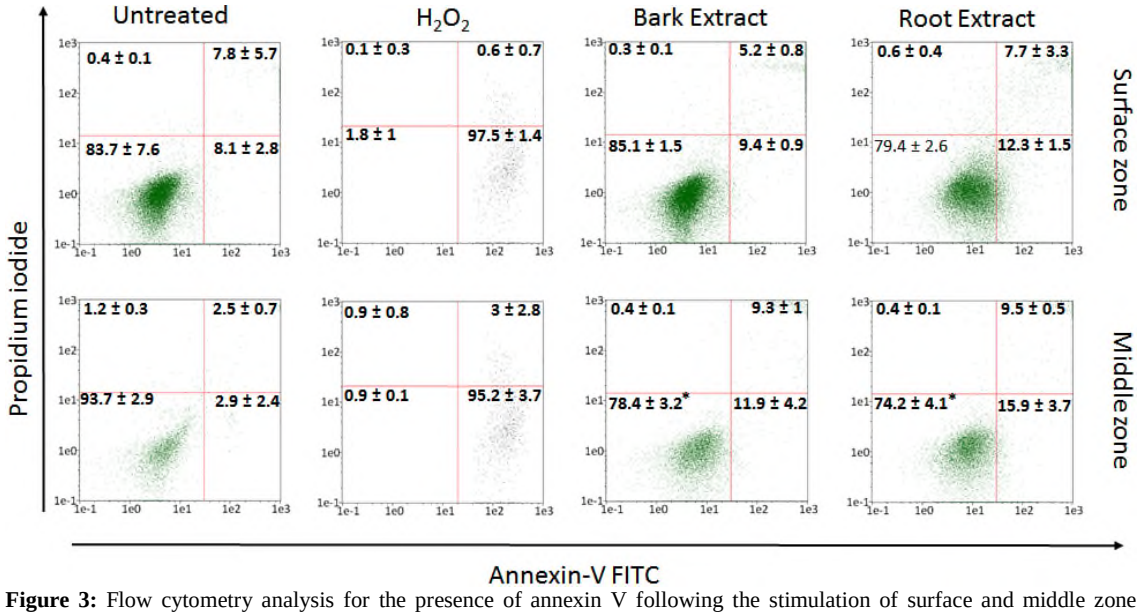

Figure 3: Flow cytometry analysis for the presence of annexin V following the stimulation of surface and middle zone chondrocytes with crude bark and root water extracts from *P. angolensis* at a concentration of 50 µg/ml. Results are in logarithmic fluorescence intensity with the x-axis (annexin V FITC) and y-axis (Propidium Iodide). The four quadrants represent necrotic cells upper left corner, late apoptotic cells upper right corner, early apoptotic cells lower right corner and viable cells lower left corner. The values in each quadrant are the mean ± SE percentage from triplicate experiments. The figure is representative of three experiments. * indicates means that had a $p < 0.05$ in comparison to the untreated chondrocytes.

P. angolensis crude water extracts induced collagen type II secretion in both superficial and middle zone chondrocytes

Superficial and middles zone chondrocytes were stimulated with crude bark and root water extracts of *P. angolensis*. After 96 hours of stimulation the tissue culture medium was analyzed for the presence of collagen type II using an ELISA. There was an increase in the amount of collagen type II secreted by both the superficial and middle zone chondrocytes in response to both the bark and root crude plant water extracts. In the superficial chondrocytes a directly proportional relationship between the amount of collagen type II detected in the tissue culture medium and the concentration of the crude bark extract was observed (Figure 4a). An inversely proportional relationship between collagen type II detected in the tissue culture medium and the concentration of the crude root extract was observed in the superficial zone chondrocytes (Figure 4a). The middle zone chondrocytes secreted the highest amount of collagen type II when stimulated with crude bark and root extracts at a concentration of 30 µg/ml (Figure 4b). Superficial zone chondrocytes produced more collagen type II when stimulated with the crude root water extract as opposed to the crude bark water extract. However, the difference in the means of collagen type II resulting from the stimulation of the superficial zone chondrocytes with crude bark or root water extracts was only significant (p=0.031) when a concentration of 30 µg/ml was used. The middle zone chondrocytes, unlike the superficial zone chondrocytes, produced more collagen type II when stimulated with crude bark water extract in comparison to the crude root water extract. A significant difference (p=0.02) in the means of collagen type II secreted by the middle zone chondrocytes in response to crude bark and root water extracts was only observed at a concentration of 15 µg/ml.

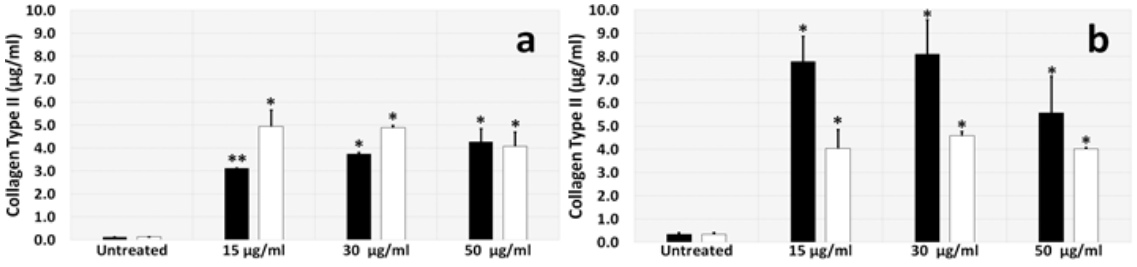

Figure 4: Collagen type II ELISA of surface (a) and middle (b) zone chondrocytes stimulated with crude bark (black bars) and root (white bars) extracts from *P. angolensis* at different concentrations. Untreated and H_2O_2-treated chondrocytes were used as controls. Error bars indicate the standard error of means from duplicate experiments. * indicates means that had a $p < 0.05$ in comparison to the untreated chondrocytes. ** indicates means that had a $p < 0.001$ in comparison to the untreated chondrocytes.

***P. angolensis* crude root water extract stimulated the development of articular cartilage matrix**

5 µm thick cartilage tissue sections were subjected to H&E as well as safranin-O staining. This was followed by a microscopic observation for the presence of the proteoglycan content, as well as chondrocyte clusters. H&E stained the cartilage matrix pink and the nucleus blue (Figure 5). The treated sections stained with H&E had more nuclei emerging from the deeper zone in comparison to the control (Figure 5c and d). Safranin-O-stained control sections exhibited smaller, round and flattened chondrocytes in comparison to the larger chondrocytes developing from the deeper zone of the treated sections (Figure 5a and b). The diameter of the pericellular matrix on safranin-O-stained sections was highest in the lower zone of the cartilage, followed by the middle and upper zone (Figure 5a and b).

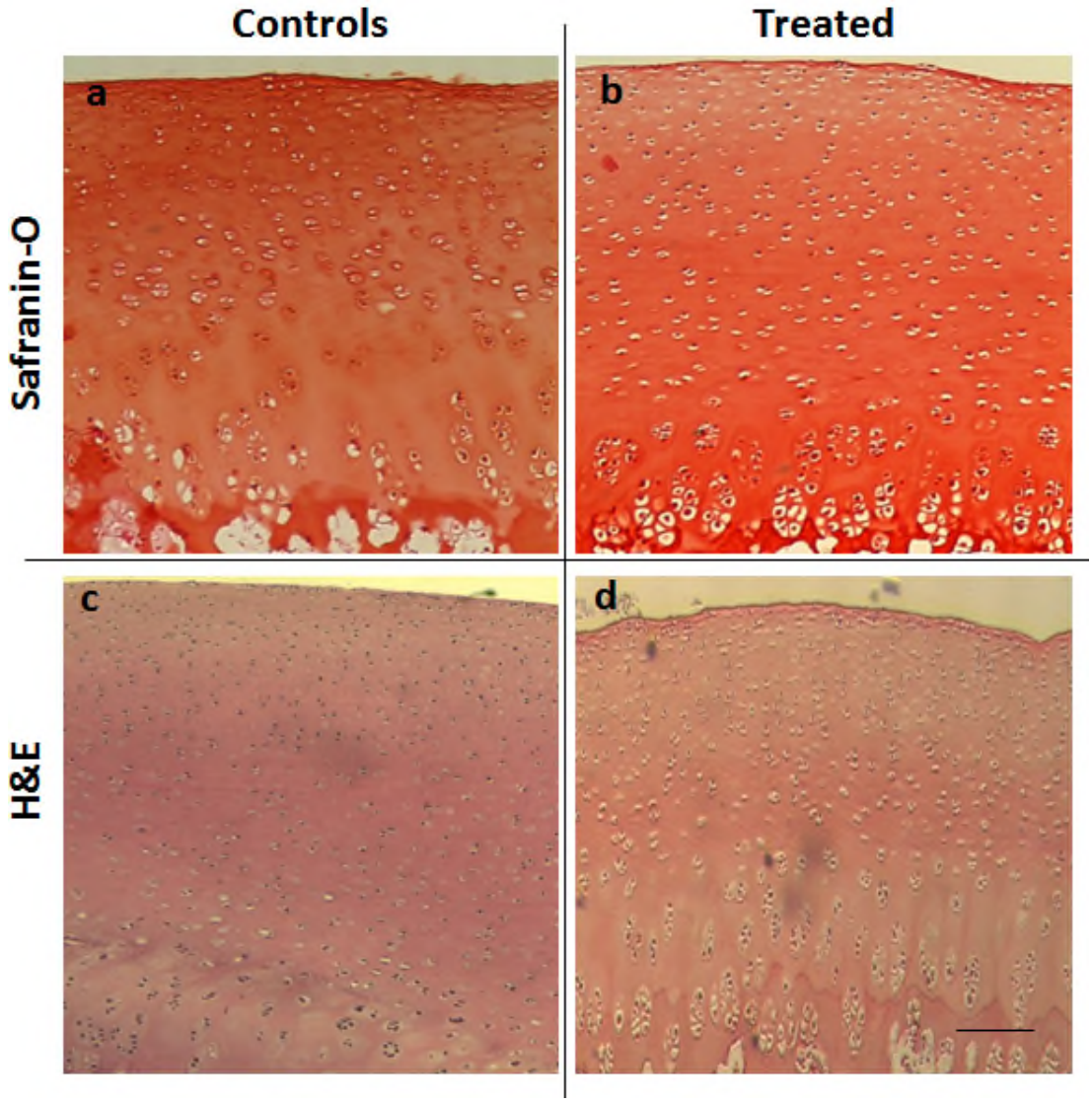

Figure 5: Histological analysis of tissue sections stained with safranin-O (a and b) and H&E (c and d). The tissue sections were treated with crude root water extracts from *P. angolensis* at a concentration of 15 µg/ml for 96 hours. Magnification (10X), scale bar = 200 µm.

***P. angolensis* crude root water extract induced the secretion and localization of collagen type II within the articular cartilage matrix**

Tissue sections of 5 µm were obtained from articular cartilage explants that had been stimulated with the crude root water extract at a concentration of 15 µg/ml. The sections were then accessed for the localization of collagen type II. Following the immunohistochemistry procedure, staining along the all-inclusive cartilage tissue sections from the uppermost surface to the deep zone was observed in tissue sections cut from both the treated and untreated cartilage. However, the

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intensity of collagen type II stain was more prominent in the flattened cell layer than in the proliferative area. No staining was observed in sections were the primary antibody was absent.

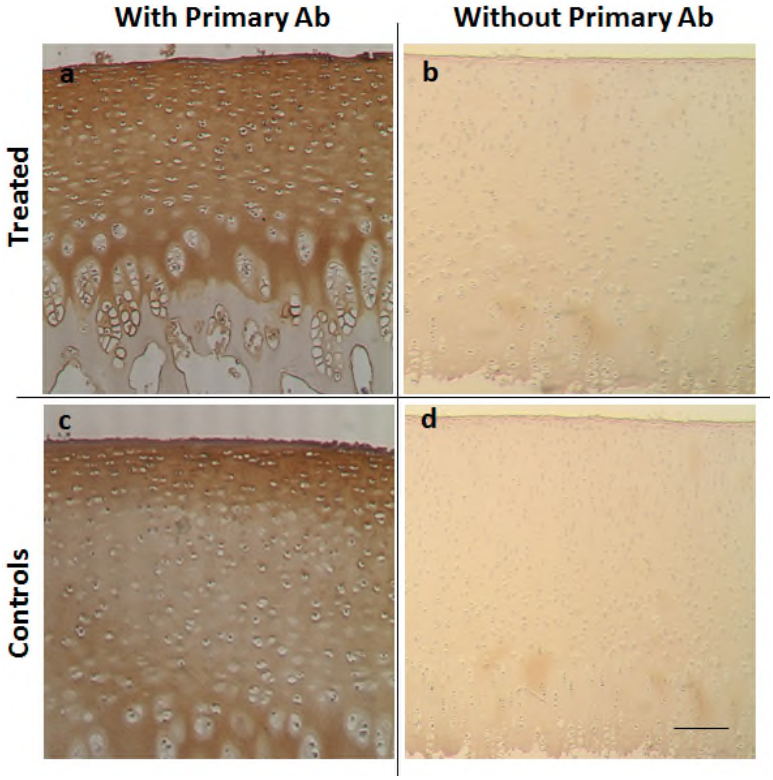

Figure 6: Immunohistochemistry analysis of tissue sections for the localization of collagen type II within the extracellular matrix in cartilage tissue. The tissue sections were treated with a crude root water extract from *P. angolensis* at a concentration of 15 µg/ml for 96 hours. Magnification (10X), scale bar = 200 µm.

Discussion

Plant-derived medicines have been used and are still being used in various communities around the world to treat medical conditions. Extracts from *P. angolensis* are being used by communities in the Sub-Saharan African region to treat diseases such as malaria, gonorrhea and ringworm, as well as conditions such as poor breast milk supply and inflammation (Geldenhuys, 2013; van Wyk & van Wyk, 2013). However, the intrinsic mechanisms through which *P. angolensis* is able to achieve its healing capabilities remain unknown. In this study we investigated the ability of the bark and root water extracts of *P. angolensis* to induce the expression of collagen type II protein by articular chondrocytes *in vitro*.

Following the stimulation of both the superficial and middle zone chondrocytes with crude bark and root extracts from *P. angolensis*, microscopic observations showed the development of cells with an elliptical/spheroidal shape attached to the bottom of the culture plates. Chondrocytes have been reported to have an elliptical/spheroidal shape (Murray et al., 2010). Therefore, the cells that were attached to the bottom of the culture plates were chondrocytes. The viability of the chondrocytes following their stimulation with the crude *P. angolensis* extracts was examined. The results showed that neither the crude bark nor root extract induced a significant increase or decrease in the viability of the superficial zone chondrocytes. A similar result was observed when the middle zone chondrocytes were stimulated with both the root and bark crude extracts at 15 and 30 µg/ml. These results show that that crude plant extracts were not toxic to the superficial or middle zone chondrocytes. However, the crude plant extracts at 50 µg/ml were toxic to the middle zone chondrocytes because at this concentration a significant reduction in the viability of these chondrocytes was observed. The loss of viability following the incubation of the middle zone chondrocytes with the crude plant extracts at 50 µg/ml could have been a result of apoptosis. When the chondrocytes were stimulated with the crude bark or root plant extracts, over 20% of the middle zone chondrocytes expressed annexin V protein on their cell membranes. The expression of annexin V protein has been used as an indication of a cell undergoing apoptosis (Figge et al., 2014; Kain & Ma, 1999). The presence of annexin V protein on the middle zone chondrocytes following their stimulation with the plant extract was an indication that *P. angolensis* at a concentration of 50 µg/ml induced apoptosis. The induction of apoptosis was an indication of the potential of *P. angolensis* extracts to be toxic when administered at a high concentration.

Following the viability assessment, the crude plant extracts were investigated for their ability to induce both the superficial and middle zone chondrocytes to secrete collagen type II. The ELISA results (figure 4) showed that both the crude bark and root extracts were able to induce a significant increase in the production and secretion of collagen type II. A higher production of collagen type II was observed in the middle zone chondrocytes (Figure 4b) in comparison to that observed in the superficial zone chondrocytes (Figure 4a). These findings were also confirmed by the immunohistochemistry results. The immunohistochemistry results showed a greater localization of collagen type II within the extracellular matrix of

the treated cartilage tissue as opposed to that of the untreated tissue (Figure 6). The intensity of collagen type II in the extracellular matrix of the treated cartilage was greater at the bottom layer than on the top layer of the cartilage tissue. These results confirm the findings of other studies, showing that collagen in cartilage tissue is mostly produced by middle zone chondrocytes (Grimmer et al., 2006; Sophia Fox et al., 2009).

Besides the up-regulation in the synthesis of collagen type II, the crude water root extract from *P. angolensis* was able to induce an increase in chondrogenesis. Chondrogenesis can be defined as the process through which cartilage is produced from mesenchyme tissue that has differentiated into chondrocytes (Goldring, 2012). When the cartilage tissue was stimulated with the crude root plant extracts at the lowest concentration there was an increase in the number of cell clusters emerging from the bottom of the cartilage tissue (Figure 5d) as opposed to those from the untreated cartilage tissue (Figure 5c). These cell clusters observed following the H&E staining were chondrocytes. Furthermore, the crude water root extract from P. *angolensis* also up-regulated the synthesis of proteoglycan. This observation was made following the staining of the cartilage tissue with safranin-O (Figure 5b). Safranin-O stain has been used as an indicator for the presence of proteoglycan in cartilage tissue (Sun et al., 2012). Aggrecan is the most abundant proteoglycan found in cartilage tissue (Toyoda et al., 2003; Watanabe et al., 1998). Therefore, since the crude water root extract from *P. angolensis* up-regulated the expression of proteoglycan, there was increased expression of aggrecan.

In conclusion, the findings of this study are of great importance in understanding the mechanisms through which *P. angolensis* enables the healing of breached tissue. The aim of this study was to investigate the ability of bark and root water extracts of *P. angolensis* to induce the expression of collagen type II protein by articular chondrocytes *in vitro*. This study showed that crude water extracts of *P. angolensis* (i) induced the proliferation of chondrocytes *in vitro,* (ii) were not toxic to either the superficial or middle zone and chondrocytes at low concentrations, (iii) induced an increase in chondrogenesis in cartilage tissue at a low concentration, (iv) induced a significant increase in the secretion of collagen type II in the extracellular matrix by chondrocytes in both the superficial and middle zone and (v) induced an increase in the secretion of proteoglycans within the extracellular matrix. These results show that extracts from *P. angolensis* could play a key role in the preparation of chondrocytes for the regeneration of torn or defective tissue through tissue engineering. However, more work is needed to identify compounds and/or molecules that are responsible for healing attributes.

Acknowledgements

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