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Full Length Research Paper

A Biflavonoid and a Carotenoid from *Rhus leptodictya*: Isolation, Characterization and Antibacterial Properties

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Acetone extracts of the leaves of *Rhus Leptodictya*were studied for antibacterial activity using bioautography and micro titre plate assay techniques against four nosocomial bacterial pathogens - *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*. A bioassay guided fractionation showed that the activity was concentrated in the carbon tetrachloride fraction. A bioautography fingerprint showed that three major compounds were responsible for the antibacterial activity. Two of these compounds were isolated by a combination of solvent - solvent fractionation and chromatographic techniques. They were characterised by ^{1}HNMR, ^{13}CNMR and 2D-NMR as 2,3-dihydro-amentoflavone, a biflavonoid andlutein, a carotenoid. This is the first report on the isolation of bioactive compounds from *Rhus leptodictya*.The four test organisms were sensitive to the two bioactive compounds. We found that Gram-positive organisms were more sensitive to 2,3-dihydro-amentoflavone with MIC values of 0.03 and 0.02 mg/ml to *E. faecalis* and *S. aureus*, respectively; while Gram-negative organisms were more sensitive to Lutein with MIC values of 0.06 and 0.09 to *P. aeruginosa* and *E. coli*, respectively. The cytotoxicity was determined using a methyl thiazole tetrazolium (MTT) based colorimetric assay against Vero monkey kidney cells. The MTT assay indicated that the LC_{50} of 2,3-dihydroxy-amentoflavone to Vero cells was 9.4 µg/ml while the LC_{50} of lutein was 9.8 µg/ml. Therefore, while the two compounds were very active against test organisms of nosocomial origin, they were also quite toxic.

Key words: *Rhus leptodictya*, bioautography, 2,3-dihydro-amentoflavone, lutein, anacardiaceae.

INTRODUCTION

The emergence of antibiotic resistance by bacteria has made the development of new microbial compounds for resistant organisms critically important (Martini and Eloff, 1998). Plant-derived medicines have been part of traditional health care in most parts of the world for thousands of years and there is increasing interest in plants as a source of agents to fight microbial diseases (Palombo et al., 2001). Medicinal and poisonous plants have always played an important role in African society.

More than 80% of the population in developing countries depends on plants for their medicinal needs. Traditions of collecting, processing and applying plants and plant-based medication have been handed down from generation to generation (Fyhrquist et al., 2001). Plants have provided and will continue to provide not only directly usable drugs, but also a great variety of chemical compounds that can be used as starting points for the synthesis of new drugs with improved pharmacological properties (Ballabh, 2008); hence the need to continuously study plants for their bioactive components and the toxic effects thereof.

Rhus leptodictya is a member of the economically im-

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portant mango family, which has about 200 species worldwide. It has about 75 to 80 trees and shrubs in southern Africa, which makes it one of the largest tree families in the region. The common names for *R. leptodictya*are mountain karee, rock karee (English); bergkaree, klipkaree (Afrikaans); inHlangushane (siSwati); Mohlwehlwe (Northern Sotho). The Manyika people use powdered roots *of R. leptodictya*for acute pain in the chest and abdominal areas. The Xhosa people use roots for gall sickness in cattle. Branch lotion and smoke from burning is used for eye complaints by Swati people (Hutchings et al., 1996). Pillay (2008) previously reported anticancer potential of this plant. Apart from the local applications for various infections, there has been no previous scientific report of the antimicrobial activities of this plant; also there is no previous report of the isolation of any compound from this plant; hence the need for the current study. We report here for the first time the antimicrobial assessment and the isolation of antibacterial compounds from *R. leptodictya*.

The leaves of *R. leptodictya* had previously been screened for anticancer activity at the Council for Scientific and Industrial Research (CSIR) in Pretoriaamong several other plants species (Fouche et al., 2008). Most of the plants showedmoderate anticancer activity with total growth inhibition (TGI) being observed at concentrations of between 6.25 and15 µg/ml. The TGI for the leaves of *R. leptodictya* against renal TK10, breast MCF7 and melanoma UACC62 cell lines were were 8.72, 55.42 and 9.83 µg/ml, respectively. The study focused on anticancer activity and there were no isolated compounds reported.

MATERIALS AND METHODS

Thin layerchromatographic plates(TLC; Kieselgel60 F_{254}) were purchased fromMerck. The following solvents and chemicalswere obtained from Sigma-Aldrich as analytical reagents: berberine chloride, benzene, ethanol, ammoniumhydroxide, ethylacetate, methanol, chloroform, formicacid, acetone, vanillin,sulphuricacid, the *p*-iodonitrotetrazolium violetreagent and the gentamicin standard. The orbital shaker used was an MRC with twelve positions for 250 to 500 ml Erlenmeyer flasks, purchased from Labotec. The Büchner funnel was a Corning and the filter paper was a Whatman No. 1. The rotary evaporator was a Buchi R-114 fitted with a diagonally sloping water-cooled condenser. The autoclave was an Optima B class from Prestige Medical. The microplate reader was a Versamax. The water was triple distilled and passed through a deionising column before use. The test bacterial strains were obtained from the National Health Laboratory Services (NHLS). Minimal Essential Medium (MEM) and Foetal Calf Serum (FCS) were obtained from Highveld Biological, South Africa.

The plant material

The leaves and twigs of *R. leptodictya* were collected from the botanical garden at the University of Pretoria in May 2008. The plant was identified by the Botanist, Ms Laurens Middleton of the Department of Botany. A voucher specimen was deposited at the herbarium of Phytomedicine Programme Department of Paraclinical Science, University of Pretoria Onderstepoort, South Africa. The plant was powdered and stored in glass storage containers maintained at ambient temperature in the dark in the Phytomedicine laboratory until used.

Extraction procedure

The powdered leaves and twigs (500 g) *of R. leptodictya* were extracted with 5 L of extraction solvent with shaking for 5 h on a Labotec shaking machine to facilitate the extraction process. The supernatant was filtered through Whatman No. 1 filter paper using a Buchner funnel and was evaporated at 40°C using a Buchi rotavapor R-114 Labotec. The extract was finally dried under a stream of cold air to dryness. After drying the weight of the extract was determined (26 g yield: 5.2%). Concentrations of 10 mg/ml were prepared in acetone for biological assays.

Thin layer chromatographic analysis of extracts

The extracts were analyzed using thin layer chromatography(TLC), Merck,Kieselgel60 F_{254} with the following solvent systems : benzene : ethanol: ammonium hydroxide (BEA)(36:4:0.4), ethylacetate : methanol : water (EMW) (40:5.4:4)and chloroform: ethylacetate:formicacid (CEF)(20:16:4).The TLC separated components were visualized as coloured spots on the plates after spraying the plates with the vanillin-sulphuric acid spray reagent (0.1 g vanillin, 28 ml methanol,1 ml sulphuric acid), followed by gently heatingthe plates to 110°C until the colour of the spots were fully developed (approximately one minute).

Bioautography assay

Four thin layer chromatography (TLC) plates were loaded with 100 µg of the extract, and dried in a stream of air before they were developed in mobile phases of varying polarities (BEA, CEF and EMW) until the solvent front reached the top of the TLC plate. The plates were dried in a stream of air. In the mean time 10 ml of a dense fresh bacterial suspension was transferred to two centrifuge tubes and centrifuged at 3500 r.p.m. for 20 min to concentrate the bacteria. The supernatant was discarded and the pellet was resuspended in 2 to 4 ml of fresh Muller Hinton (MH) broth. This was mixed on the vortex shaker and approximately 4 ml medium was then added to give the concentrated bacterial culture of each test organism. The dried plates were sprayed with the concentrated bacterial cultures of *E. coli, P. aeruginosa, S. aureus,* and *E. faecalis* until completely moist with the aid of spraying gun, and enhanced using a humidified chamber for 24 h to allow the microorganisms to grow on the plates. The plates were then sprayed with 2 mg/ml of p-iodonitrotetrazolium violet (INT) (Sigma) and incubated for 30 min. The emergence of purple-red colour resulting from the reduction of INT into the respective formazan was a positive indicator of cell viability. Clear zones were indicative of antibacterial growth activity of the extracts.

Minimal inhibitory concentration assay of the plant extracts

Minimal inhibitory concentration (MIC) is accepted as the lowest concentration of extract that inhibits growth of test organisms. The method of Eloff (1998b) was used. The assay was initiated by pouring sterile water aliquots (100 µl) into wells of microtitre plates. Exactly 100 µl of 10 mg/ml extract prepared in acetone was added in row A and mixed using a micropipette. From row A, 100µl was aspirated and added into row B and mixed with sterile water aliquots (100 µl). The procedure was repeated until all the wells were filled. An additional 100 µl in row H was discarded. Two columns

were used as control, one as sterility control (no cultures were added) and the other for growth control (the extracts were replaced with 100 µl of acetone). Concentrated suspensions of microorganisms (100 µl) were added to each well except the sterility controls. The microtitre plates were sealed in a plastic bag with a plastic film sealer (Brother) before they were incubated at 37°C in a humidified incubator for 18 h. After incubation 40 µl of 0.2 mg/ml INT was added to each well and the plates were incubated for a further 2 h before observation. The development of red/purple colour, resulting from the formation of the red/purple formazan, was indicative of growth (positive indicator of cell viability). MIC values were regarded as the lowest concentrations of the extracts that inhibit the growth of the test organisms (the well of first disappearance of the red formazan colour). Gentamicin was used as reference. The experiments were performed in triplicate.

Methyl thiazole tetrozolium-based colorimetric assay (MTT)

The two isolated compounds were tested for cytotoxicity against Vero African monkey kidney cells (Vero cells) using the method described by Mosmann (1983). Berberine chloride (Sigma) was used as positive control in this method. The intensity of colour is directly proportional to the number of surviving cells. The MTT is described in detail including the cell preparations, calibration curve and the actual cyctotoxicity testing.Tests were carried out in quadruplicate and each experiment was repeated three times.

Cell preparations

A suitable passage of cells of a subconfluent culture were harvested and centrifuged at 200 g for 5 min, and the cell pellet resuspended in growth medium to a density of 2.4×10^3 cells/ml. Minimal Essential Medium (MEM) (Highveld Biological, South Africa) supplemented with 0.1% Gentamicin (Sigma) and 10% Foetal Calf Serum (FCS) (Highveld Biological, South Africa) was used as growth medium. Cells were incubated at 37°C in a 5% CO_2 atmosphere in this growth medium under sterile conditions for 48 h. The cells were subsequently dispersed. The growth medium was then removed by centrifugation and the cells were re-suspen-ded in fresh growth medium. Cells prepared in this way were ready for use in the preparation of the calibration curve and for the cytotoxicity tests.

Calibration curve

To calibrate the method, a series of cell densities ranging from 0 to 600 cells/ml were prepared and placed in the wells of microtitre plates with 96 wells. The plateswere incubated for 48 h . A 5 mg/ml solution of INT in PBS was added to the wells and the plates were incubated for 5 h, after which no further colour development was observed. Acidic DMSO was added to dissolve the blue crystals, and to make up to a final volume of 25 ml. The absorbance was measured on a UV/Vis spectrophotometer, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and calibration settings of 0.00 to 1.00. The absorbance was plotted against cell density to give a linear calibration curve.

Cytotoxicity tests

Cell suspension (200 µl) was added into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 µl) was added into wells of columns 1 and 12. The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator, until no further colour development was observed. The medium was then removed from wells using a thin tube attached to a hypodermic needle and immediately replaced with 200 µl of test compound or berberine chloride (Sigma) (positive control) at various known concentrations. The concentration gradient of test compound and berberine chloride was obtained by quadruplicate serial dilutions prepared in growth medium. The microtitre plates containing treated and untreated cells were incubated at 37C in a 5% CO_2 incubator for 24 h. MTT (30 µl) (Sigma) (stock solution of 5 mg/ml in phosphate-buffered saline [PBS]) was added to each well and the plates incubated for a further 4 h at 37C. The medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl of DMSO to each well, followed by gentle shaking of the MTT solution. The amount of MTT reduction was measured immediately by detecting absorbance at 570 nm using a microplate reader (Versamax). The wells in column 1, containing only medium and MTT but no cells, were used to blank the reader. The LC50 values were calculated as the concentration of test compound or plant extract resulting in a 50% reduction of absorbance compared to untreated cells.

Isolation of antibacterial compounds

The dried acetone extract (26 g) was subjected to the solvent - solvent fractionation method described by Suffness and Dourous (1999) and adapted by Eloff (1998a). The bioautography fingerprint showed that two spots in the carbon tetrachloride fraction were responsible for its activity. The carbon tetrachloride extract (6 g), which had the most antibacterial compounds, was subjected to fraction collection based column chromatography on silica gel 60 (Merck). Fifty-seven fractions were collected in 100 ml test tubes. Based on TLC fingerprint, the fractions were combined to 5 fractions. Compound D1 was obtained from fraction 4 (412 mg) by precipitating with hexane. Compound D2 was obtained from Fraction 5 (669 mg) by running a second column on silica gel with a narrow gradient of Chloroform: Methanol (98:2 to 90:10). The activi-ties of these compounds on four nosocomial bacterial pathogens and their cytotoxic effects were tested using the standard proce-dures as described above.

RESULTS AND DISCUSSION

The Bioautogram in Figure 2 showed that the active compounds were mainly in the carbon tetrachloride extract. This extract showed three major active spots. Column chromatographic separation of the carbon tetrachloride fraction led to the isolation of two bioactive compounds. Compound D1 was isolated as yellowish-reddish amorphous solid. Inspection of the carbon NMR spectra showed the presence of 40 carbon resonances, with signals in the upfield and downfield region. The spectra of the isolated compound showed a characteristic pattern for carotenoids (C40 derivatives). The ^{13}C chemical shift data was compared with the ^{13}C data of previously isolated carotenoids. That the ^{13}C data for isolated compounds compared perfectly with data from the previously isolated carotenoid-lutein is shown in Table 1 (Arigoni et al., 1999). The correlation of the ^{13}CNMR data in Table 1 was considered to present conclusive identification of the compound as lutein.

The mass spectrum of compound D2 (Figure 1) shows a molecular ion at m/z 539[M - 1], which is consistent with molecular formulae of $C_{30}H_{20}0_{10}$. The fragmentation pattern is consistent with the characteristic pattern of a

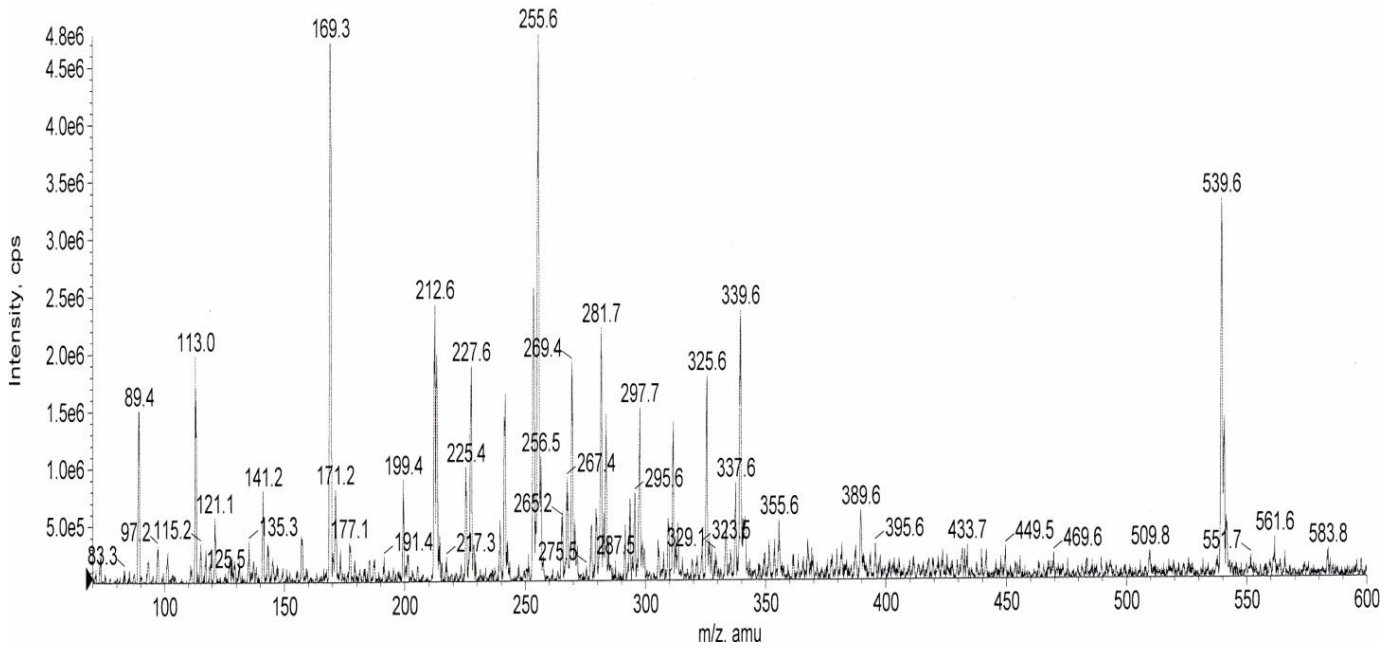


Figure 1. MS spectrum of Compound D2 .

Table 1. ^{13}C data for isolated compound compared perfectly with known data for lutein.

Carbon	Lutein	BSRL-1	Carbon	Lutein	BSRL-1	Carbon	Lutein	BSRL-1
1	37.09	37.11	7’	128.79	128.71	14	132.56	132.58
1’	34.01	34.16	8	138.47	138.49	14’	132.56	132.58
2	48.30	48.35	8’	137.68	137.56	15	130.09	130.09
2’	44.60	44.64	9	135.68	135.68	15’	130.04	130.04
3	65.04	65.09	9’	135.06	135.06	16	30.26	30.26
3’	65.89	65.94	10	131.28	131.31	16’	24.19	24.19
4	42.46	42.49	10’	130.77	130.81	17	28.72	28.68
4’	124.39	124.45	11	124.90	124.93	17’	29.50	29.46
5	126.13	126.17	11’	124.77	124.81	18	21.62	21.63
5’	137.98	137.98	12	137.53	137.56	18’	22.86	22.88
6	137.68	137.56	12’	137.53	137.56	19	12.75	12.76
6’	54.87	54.95	13	136.40	136.40	19’	13.10	13.10
7	125.53	125.59	13’	136.48	136.46	20.20’	12.81	12.81

biflavonoid, which consists of a flavanone and a flavone. The NMR spectra of compound D2 indicated two flavone units linked through the C-3 of a flavone ring to the C-8 of the second flavone. This class of compounds consists of three ring systems A, B, and C.Ring B which has a hydroxy substitution at C-4 often gives a typical 4 peak pattern of two doublets (AA’BB’ system) with a characteristic coupling constant; this pattern was clearly shown in C-3, C-5, C-2 and C-6 with resonance from 6.86 to 7.68 ppm and a coupling constant of 9 Hz at ring IIB (Figure 3). The data obtained correlate with data obtained on previous isolation of biflavonoid; 2,3-dihydro amentoflavone compound (Chen et al., 2005) (Figure 4).

Antibacterial studies

From the bioautography and MIC studies as shown in Figure 1 and Table 2, the four microorganisms are susceptible to the two compounds. The most susceptible organism to D1 is *P*. aeruginosa with MIC Value of 0.06 mg/ml, while compound D2 shows better activity against Gram-positive organisms with MIC value of 0.02 mg/ml. The activity of the isolated compounds compares well

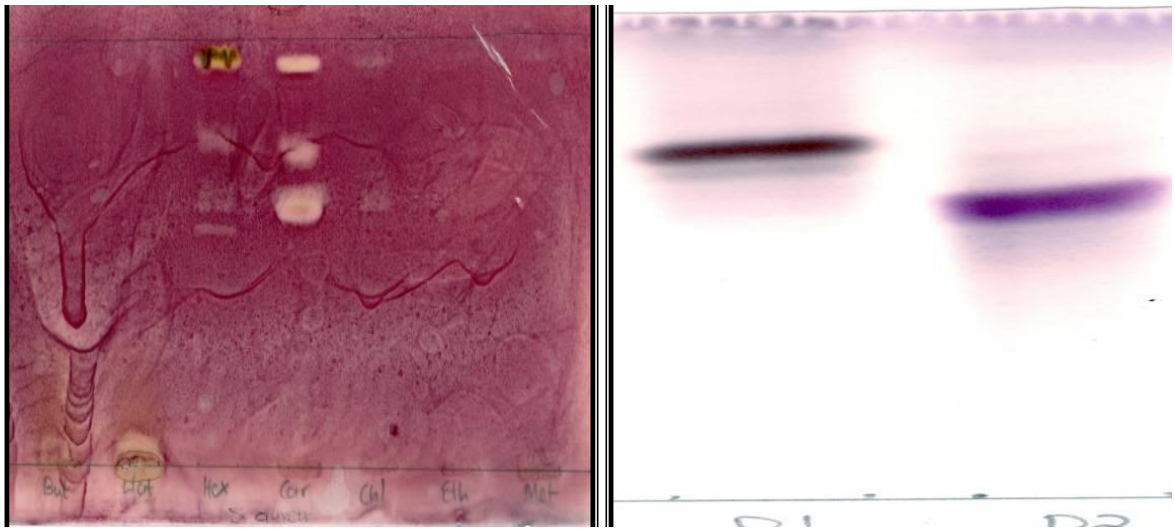


Figure 2. (a) Bioautogram of different extracts of *Rhus leptodictya.* Colorless areas denote inhibition of bacterial growth. (b) TLC chromatogram of the isolated compounds (D1 and D2).

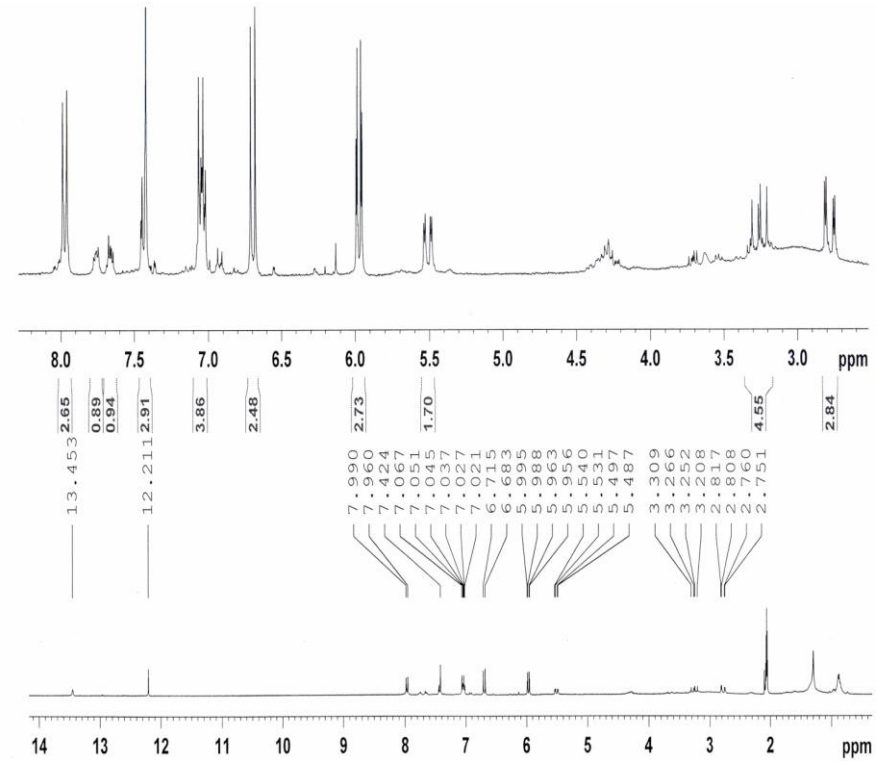
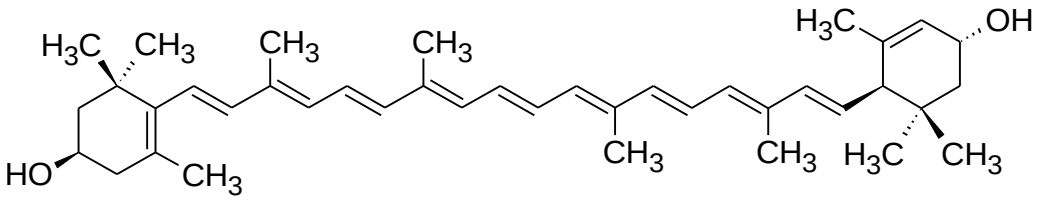


Figure 3. 1HNMR spectrum of compound D2.

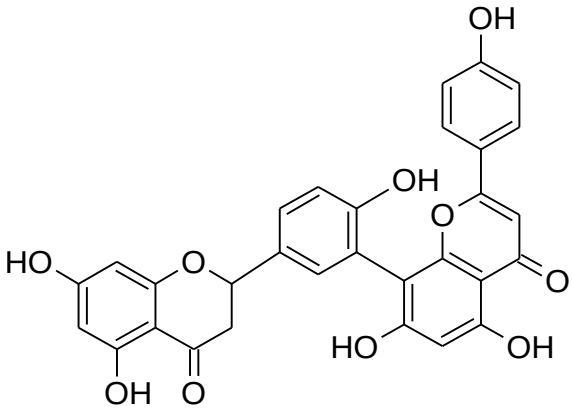
with the reference compound (Gentamicin) as shown in Table 1. This could possibly account for the local application of this plant extract forvarious infections

Toxicity studies

The compounds indicated high level of cell toxicity against the cell line with LC50 of 9.4 µg/ml for 2,3-amentoflavone and 9.8 µg/ml forlutein. While the carotenoids are known from literature to show some measure of toxicity (Leal et al., 1998); 2,3-dihydro amentoflavone can be subjected to some structure activity studies to obtain an optimum compound with minimum toxicity while maintaining its good bioactivity.



Lutein



2,3-dihydro amentoflavone

Figure 4. Compound D1: lutein, a carotenoid and D2: 2,3-dihydro amentoflavone, a biflavonoid.

Table 2. MIC values of the isolated compounds and Gentamicin control against bacterial species.

Isolated compounds	Lutein(D1) (mg/ml)	2,3-dihydro amentoflavone (D2 (mg/ml)	Gentamicin control (mg/ml)
S. aureus (+)	0.16	0.02	0.02
E. faecalis (+)	0.16	0.03	0.02
P. aeruginosa (-)	0.06	0.02	0.02
E. coli (-)	0.09	0.19	0.03

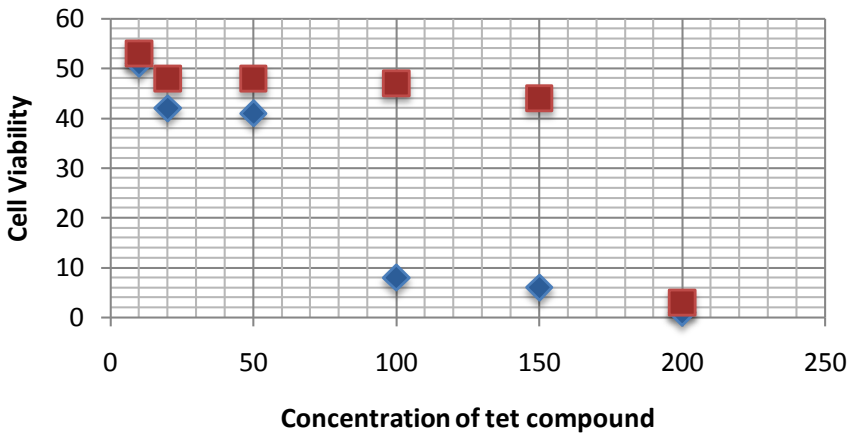


Figure 5. Dose response curve for compound D1 and D2.

The cytotoxicity dose response curve for the two compounds is shown in Figure 5.

Conclusions

Current studies report for the first time the isolation of antibacterialcompounds from the leaves of Rhus leptodictya. Of the three compounds observed in the zones of inhibition in the bioautographic fingerprint, two antibacterial compounds were isolated and identified as lutein and 2,3-dihydro amentoflavone. Four microorganisms are susceptible to the two compounds. The Gram-negative

organisms were more sensitive to lutein; while Gram-positive organisms had higher sensitivity to 2,3-dihydro amentoflavone. The activity of the isolated compounds compares well with the activity of the reference compound, Gentamicin used in this study.The compounds indicated high cell toxicity against Vero monkey kidney cell lines with LC50 of 9.4 µg/ml and 9.8 µg/ml for 2,3-amentoflavoneand luteinrespectively. While the carotenoid, luteinis known from literature for its cytotoxicity, 2,3-dihydro amentoflavone can be subjected to some structure activity studies to obtain an optimum compound with minimum toxicity while maintaining its good bioactivity. The study validated the use of this plant extract in the treatment of eye, abdominal and chest ailments of infectious origin. These compounds could be lead compounds for the development of other antibacterial pharmaceuticals.

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REFERENCES

Arigoni D, Esenreich W, Latzel C, Sagners S, Radykewicz T, Zenk MH, Bacher A (1999). Dimethylallyl pyrophosphate is not the committed preusor of isopentenyl pyrophosphate during terpenoid biosynthesis from 1-deoyxylose in higer plants. Biochemistry 96: 1309-1314.

Ballabh B, Chaurasia OP, Amed Z, Signh SB (2008). Traditional medicinal plants of cold desert Ladakh-Used against kidney and urinary disorders. J. Food Chem. 118: 331-339.

Chen J, Duh C, Chen JF (2005). New cytotoxic biflavonoids from Selaginella discatula. Planta Med. 71:659-665.

Eloff JN(1998a). Which extractant should be used for the screening and isolation of antimicrobial components from plants? Planta Med. 64:711-713.

Eloff JN (1998b). A sensitive and quick method to determine the minimum inhibitory concentration of plant extracts for bacteria. J. Ethnopharmacol. 60:1-8.

Fyhrquist P, Mwasumbi L, Haeggström CA, VuorelaH, Hiltunen R, Vuorela P(2001). Ethnobotanical and antimicrobial investigation on some species of *Terminalia* and *Combretum (*Combretaceae). J. Ethnopharmacol. 79:169-177.

Hutchings A, ScottA, Lewis A, Cunningham AB (1996). Zulu Medicinal Plants: An Inventory. University of Natal Press, Pietermaritzburg.

Leal M, Gonzalez E, Ruiz F, Shimada A (1998). Effects of carotenoids on cytotoxicity of T-2toxin chicken hepatocytes in vitro. Toxicol. In Vitro 12:133-139.

Martini N, Eloff JN(1998). The preliminary isolation of several antibacterial compounds from *Combretum erythrophyllum* (Combretaceae). J. Ethnopharmacol. 62: 255-2643.

Mosmann T(1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. J. Immunol. Methods. 65: 55-63.

Palombo EA, Semple SJ (2001). Antibacterial activity of traditional Australian medicinal plants. J. Ethnopharmacol. 77: 151-157.

Fouche G, Cragg GM, Pillay P, Kolesnikova N, Maharaj VJ, Senabe J (2008). In vitro anticancer screening of South African plants. J. Ethnopharmacol.119(3): 455-461.