

Phytochemical Profiling, Antioxidant and Antibacterial Activities of Leaf Extracts from *Rhus leptodictya*

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ABSTRACT

An integral part of nature is herbal plants which contain natural substance essential to promote health. The focal point of this research resides on the phytochemical, antioxidant and antibacterial investigations of *Rhus leptodictya* leaf extract. Hexane, dichloromethane, ethyl acetate, acetone, and methanol solvent extracts were tested for their antioxidant and antimicrobial properties. Phytochemical analysis of *R. leptodictya* extracts revealed the presence of phenols, tannins, saponins, and flavonoids. Acetone extract with total flavonoid contents of 18.22 ± 2.1 mg/g while methanol extract with phenol contents (0.38 ± 0.002 mg/g) exhibited the highest amount amongst the studied extracts in the preliminary phytochemicals screening. The antioxidant activity of the plant extracts was evaluated using TLC based standard qualitative 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The methanol and acetone extracts showed maximum antioxidant bands compared to other selected solvents and promising radical scavenging activity at par with the standard antioxidant ascorbic acid ($IC_{50} = 0.125 \pm 0.013$ mg/ml). The bioautography assay also demonstrated the bacterial growth inhibition of active compounds present in leave extracts. Acetone and ethyl acetate fractions showed the highest number of antibacterial active compounds against *S. aureus*. Minimum inhibitory concentration of the extracts examined using microplates of 96 wells containing Muller-Hinton methods against some bacterial strains, revealed methanol and acetone fractions with the lowest MIC value of 0.04 mg/ml against *S. pneumoniae*, while methanol extract possesses 0.008 mg/ml against *S. aureus* and *B. subtilis*. Results from this study project the *R. leptodictya* leave extracts constituents as health supplements that could be useful in food industries and in the treatment of various infectious diseases.

Keywords: *Rhus leptodictya*, Phenolic, Tannins, Total flavonoids, Antibacterial, Antioxidant, Bioautography.

INTRODUCTION

The increasing incidence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced responsiveness to antibiotics have raised serious concern in health delivery and accessibility due to untreatable bacterial infections^{1,2}. There is, therefore, the needed to search for new antimicrobial agents. Plants are the important source of potentially useful structures for developing new chemotherapeutic agents³. Since time immemorial, plants have been used in the preparation of drugs thus, act as sources of medicine.

Medicinal plants are well known natural sources for the treatment of various diseases since antiquity. Natural product, either pure compounds or standardized plant extract, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. In a sense, it forms the basis of all medicine, the mother of all remedy used today. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties⁴. They are natural bioactive compounds such as primary and secondary metabolites encompassing a wide array of functions found in medicinal plants⁵. The most common plant secondary metabolites

include the following groups: alkaloids, anthraquinones, coumarins, essential oils, flavonoids, steroids and terpenoids^{4,5}.

The World Health Organization (WHO) estimates that 80 % of the people living in developing countries almost exclusively use traditional medicine⁶. Different parts of the plant are used in the indigenous system of medicine for the treatment of various human ailments such as wounds, ulcers, bowels, and cholera⁷. The leaves of *Rhus* plants have been used in the treatment of wounds, abscesses and externally for the treatment of neuralgia, throat infections and a wide range of skin diseases such as ringworm, ulcers, and rashes. Some part of *Rhus* species is used in South Africa for the treatment of epilepsy⁸.

Rhus leptodictya is commonly known as mountain karee, rock karee, in English: bergkaree, klipkaree in Afrikaans; Mohlwehlwe in Sotho and Inhlanguwane in Siswati. It is a large shrub tree native in South Africa. The leaves are divided into three leaflets, bright green with toothed margins and can grow up to nine meters but usually rather a shrubby bush of about three to four meters. These plants are distributed in four Northern Province of South Africa and other surrounding countries such as Zimbabwe.

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Mozambique, Lesotho, Botswana. The mountain karee grows naturally in a variety of habitat types including woodland, forest margins and bushveld^{9,10}.

Efforts have been made by researchers to date on *Rhus* extracts with an indication as a promising potential to providing renewable bio-products with the following excellent bioactivities: Antifibrogenic, anti-inflammatory, antioxidant, antimalarial, antimicrobial, antimutagenic, antithrombin, antifungal, cytotoxic, antitumorigenic, antiviral, leukopenia, and hypoglycemic^{11,12,13}. Six bioflavonoids such as robustaflavone, amentoflavone, agathisflavone, volkensiflavanone, succedaneaflavanone, and rhusflavonone are compound isolated from *Rhus succedanea* seeds and tested for inhibitory activities against a number of viruses including respiratory viruses¹⁴. The extracts of *Rhus pyroides* containing two bioflavonoids: agathisflavone and amentoflavone with activity in the H-Ro 15-1788 (flumazenil) binding assay were isolated by high pressure liquid chromatography (HPLC) from fractionation of ethanol extract of *R. pyroides* leaves¹⁵.

Antioxidant defense mechanisms including antioxidant enzyme-systems are significant for cells and tissues protection against oxidative damage caused by reactive oxygen species (ROS)^{16,17}. Hence, substances acting as antioxidants are molecules that inhibit or quench free radical reactions and delay or inhibit cellular damage¹⁷. Free radicals exist in the form of singlet oxygen (ROS), hydroxyl radical and superoxide anions¹⁸. Oxidative stress plays a key role in the development of chronic and degenerative ailments including cancer, aging, autoimmune disorders, arthritis, cardiovascular and neurodegenerative diseases¹⁶.

The study aimed at preparing various crude extracts using different of solvent polarities and quantitatively evaluating their total phenol, flavonoids contents, antioxidant and antibacterial potential *R. leptodictya* leaf material collected from University of Pretoria (Onderstepoort campus), South Africa.

MATERIALS AND METHODS

Plant collection

The leaves of *Rhus leptodictya* were collected from the University of Pretoria (Onderstepoort campus), South Africa. The plant samples were identified taxonomically and authenticated by Prof. Eloff (University of Pretoria). The leaves were washed with running water and dried at room temperature for three weeks. The dried leaf material was pulverized using Macasalab mill (model 200 Lab), to a fine powder and stored in an air-tight container until time of extraction.

Extraction procedure

Different aliquots of 3 g of the fine powder materials of *R. leptodictya* were extracted with 30 ml of five different solvents of increasing polarity (hexane, dichloromethane, ethyl acetate, acetone, and methanol, Merck, South Africa) in a 50 ml centrifuge tubes by maceration for about 5 h. The extracts were then centrifuged using an EBA 30 Hetch centrifuge at 6000 rpm for 20 minutes and the supernatant filtered through Whatman No.1 filter paper

into pre-weigh glass vials and placed under a stream of cold air to dryness and the mass of the powdered extracts determined.

Thin layer chromatography (TLC) analysis

The chemical profile of the plant extracts was determined by thin layer chromatography (TLC) using aluminum-backed TLC plates (Merck, silica gel 60, F₂₅₄). The extracted plant materials were re-dissolved in acetone to obtain a final concentration of 10 mg/ml, and 10 µl of the extracts were spotted on the TLC plates. Three solvents system were used to develop the plates, in the form: Ethyl acetate/ methanol/ water (40:5:1) [EMW] (polar neutral); Chloroform/ ethyl acetate/ formic acid (5:4:1) [CEF] (intermediate polarity/acidic); Benzene/ ethanol/ ammonium hydroxide (90:10:1) [BEA] (non-polar/ basic). The development of the chromatogram was achieved in a closed TLC developing tank in which the atmosphere has been saturated with the eluent. Samples were applied rapidly and developed without delay to minimize the possibility of oxidation or photo-oxidation of constituents. The developed TLC plates were visualized under UV light at 254 and 365 nm to detect UV absorbing or fluorescing bands. The plates were then sprayed with vanillin spray reagent prepared by dissolving 0.1 g of vanillin (Sigma) in 28 ml of methanol; 1ml of sulphuric acid and heated at 110° C to optimal color development^{19,20}.

Phytochemical analysis of extracts

Qualitative analysis

Chemical tests for the screening and identification of bioactive chemical constituents were carried out on the extracts using the standard procedures described by Bhatnagar and co-workers²¹. The test was carried out to detect the presence of metabolites such as alkaloids, glycosides, terpenoids and steroids, flavonoids, reducing sugar, saponins, and tannins.

Quantitative analysis

Determination of total phenol content

Total phenol content was determined by Folin-Ciocalteu reagent method as previously described with modification^{22,23}. From each solvent extracts (1 mg) was dissolved in methanol (1 ml). A total of 10 % Folin-Ciocalteu reagent was prepared by adding Folin-Ciocalteu reagent (10 ml) in water (90 ml). Then, 5 % sodium carbonate was prepared by dissolving Na₂CO₃ (3 g) in water (50 ml). Each crude sample (200 µl) was taken in a test tube, 10 % Folin-Ciocalteu reagent (1.5 ml) added. Then all the test tube was allowed to stand in the dark for 5 min. Finally, 5 % Na₂CO₃ (1.5 ml) was added to the solution and mixed by vortex. Furthermore, the test tubes were kept again in the dark for 2 h. The absorbance measured for all solution using UV-spectrophotometer at constant wavelength 750 nm (UV-Vis model: T80+, PG Instruments Ltd). Gallic acid standard curve was prepared by dissolving gallic acid (3 mg) in methanol (10 ml) to give a concentration of 300 mg/l. The working standards were obtained by adding methanol to give a serial concentration of 200, 100, 50, 25 and 0 mg/l. The absorbance of all the solutions was measured spectrophotometrically at constant wavelength 750 nm. Total phenol values were expressed

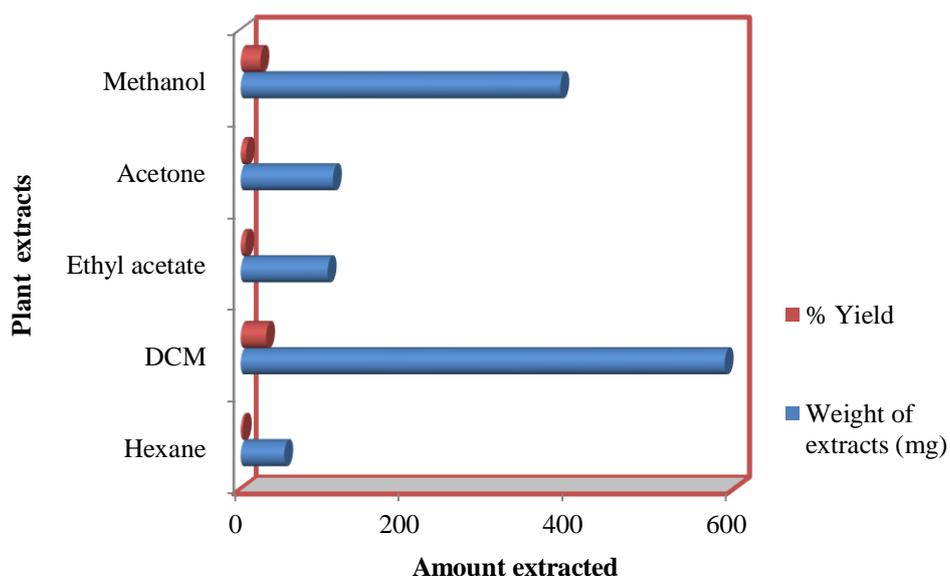


Figure 1: Total amount extracted 3 g powered leaves of *Rhus leptodictya*.

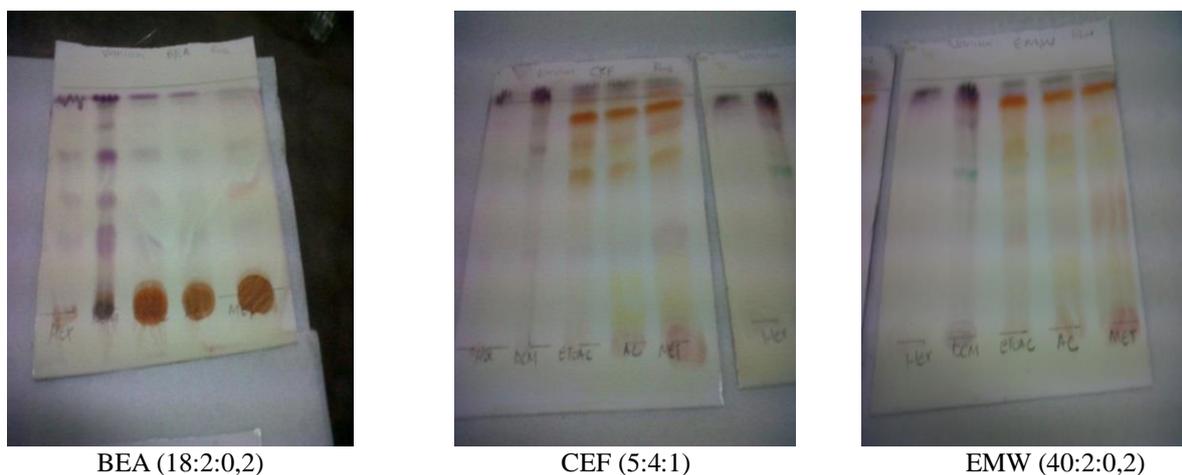


Figure 2: Chromatograms of the hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), acetone (Ac) and methanol (MeOH) under different solvent systems sprayed with vanillin.

in terms of gallic acid equivalent (mg/g of plant material), a common reference compound. Results were calculated from the mean of three replicates.

Determination of total flavonoids content

The method used was the aluminum chloride calorimetric method with some modification²⁴. 1 ml of each methanol, acetone, ethyl acetate, dichloromethane and hexane extract in methanol was mixed 3 ml of methanol, 0.2 ml of 10 % aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and remained at room temperature for 30 minutes. The absorbance was measured at 420 nm (UV-Vis model: T80+, PG Instruments Ltd). Quercetin was used for the calibration curve- five different concentrations: 12.5, 25, 50, 75, 100 ppm. Flavonoids contents were determined from the standard curve and were expressed as quercetin equivalent. Results were calculated from the mean of three replicates.

TLC based qualitative analysis for Antioxidants by *Rhus* extracts

The method used for measuring antioxidant is the one that involves the generation of free radical species. An atom or molecule that has at least one unpaired electron is called free radical. They generally abstract electrons from other molecules, thereby inducing a chain reaction of electron abstraction and radical formation^{16,17}.

The free radical scavenging was carried out using the 2,2-diphenyl-1-picrylhydrazyl by TLC-DPPH method. The DPPH radical is reduced from a stable free radical, which is purple in color to diphenylpicryl hydrazine, a yellow colour. Different extracts of *R. leptodictya* were separated by TLC in a different solvent system, air dried and then sprayed with 0.2 % of DPPH in methanol. The chromatogram was examined for colour change over 30 min in dark environment. The presence of antioxidant compounds was detected by the colour change from purple

Table 1: R_f values of different compounds from TLC Profile in different solvent systems.

Solvent systems	Hexane	Dichloromethane	Ethyl acetate	Acetone	Methanol
BEA (18:2:0.2)	0.35	0.35	-	-	-
	0.434	0.434	-	-	0.434
	0.652	0.652	-	-	-
	-	0.797	-	-	-
EMW (40:5:4.5)	-	0.89	0.89	0.89	-
	-	-	0.366	-	-
	0.976	0.563	0.563	-	-
	-	-	0.648	-	-
CEF (5:4:1)	-	0.887	0.815	-	-
	-	0.743	0.944	-	-
	-	0.878	0.622	0.243	0.333
	-	-	0.743	-	0.432
			0.797	0.797	
			0.919	0.919	0.919
			-	0.959	0.959

BEA = Benzene/ Ethanol/ Ammonia; EMW = Ethyl acetate/ Methanol/ Water
 CEF = Chloroform/ Ethyl acetate/ Formic acid

Table 2: Phytochemical constituents of the leaves of *R. leptodictya*.

Plant extracts	Phenols	Tannins	Saponins	Flavonoids
Hexane	+	-	+	+
Dichloromethane	+	-	+	+
Ethyl acetate	+	+	+	+
Acetone	+	+	+	+
Methanol	+	+	+	+

+ = present - = absent

Table 3: Total phenol and flavonoid contents in *R. leptodictya*.

Extract	Total Phenolic (mg/g)	Total Flavonoids (mg/g)
Methanol	0.38 ± 0.002	11.54 ± 1.7
Acetone	0.21 ± 0.007	18.22 ± 2.1
Ethyl acetate	0.18 ± 0.002	8.30 ± 1.4
Dichloromethane	0.003 ± 0.001	10.03 ± 3.3
Hexane	0.12 ± 0.001	2.35 ± 0.2

Table 4: Radical scavenging activities of solvent fractions of *R. leptodictya* leave (IC₅₀).

Extracts	DPPH radical scavenging (mg/ml)
Ethyl acetate	0.328 ± 0.041
Hexane	0.585 ± 0.182
Dichloromethane	0.511 ± 0.190
Acetone	0.134 ± 0.018
Methanol	0.212 ± 0.076
Ascorbic Acid*	0.125 ± 0.013

* = Standard

(background) to yellow spots. The DPPH free radical scavenging activity of the sample was also quantified spectrophotometrically at 517 nm wavelength (UV-Vis model: T80+, PG Instruments Ltd) by standard protocol²⁵. Obtained results were reported as the IC₅₀ (the amount of

antioxidant essential to decrease the initial DPPH concentration by 50 %). Ascorbic acid was used as positive control.

Biological assays for screening

Bacterial culture and growth conditions

Four different organisms were used for the screening tests; the organisms were obtained from the biotechnology laboratory at the Vaal University of Technology. The organisms used for the assays includes: *Staphylococcus aureus* [gram-positive, ATCC 29213], *Streptococcus pneumoniae* [gram-positive, ATCC 49619], *Escherichia coli* (gram-negative, ATCC 25922), *Bacillus subtilis* [gram-positive, ATCC 19659]. The cultures were maintained on Muller Hinton (MH) agar at 4 °C and sub-cultured on to nutrient broth at 37 °C for 18 h prior to bioautography and MIC assay.

Bioautography assay of the extracts

The TLC plates were developed for the bioautography against *S. aureus* as previously described²⁶. The plates were loaded with 10 µl of each extract, dried in the stream of air before developing in different mobile phase (chloroform/ethyl acetate/formic acid [CEF] 5:4:1). The developed TLC plates were dried in a stream of air to allow the solvent to evaporate before spraying with an actively growing bacteria culture: *S. aureus* [ATCC 29213]. The chromatograms were then incubated for 24 h at 37 °C under 100% relative humidity to allow optimal microorganism growth. After 24 h incubation, the bioautograms were sprayed with an aqueous solution of 2 g/ml of *p*-Iodonitrotetrazolium (INT) violet (Sigma-

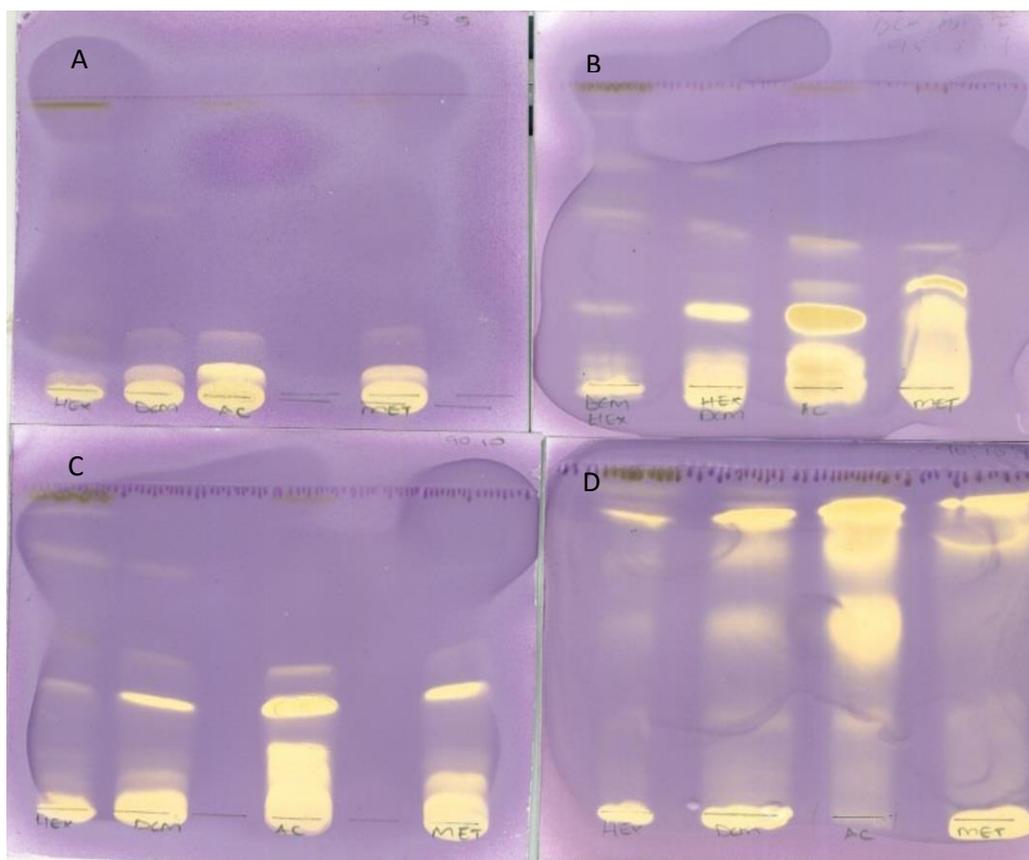


Figure 3: Chromatograms of antioxidant activity sprayed with DPPH in a different solvent system. A: Hexane: Ethyl acetate (95:5) B: Hexane: Ethyl acetate (90:10) C: Hexane: Ethyl acetate (85:15) D: Hexane: Ethyl acetate (80:20).

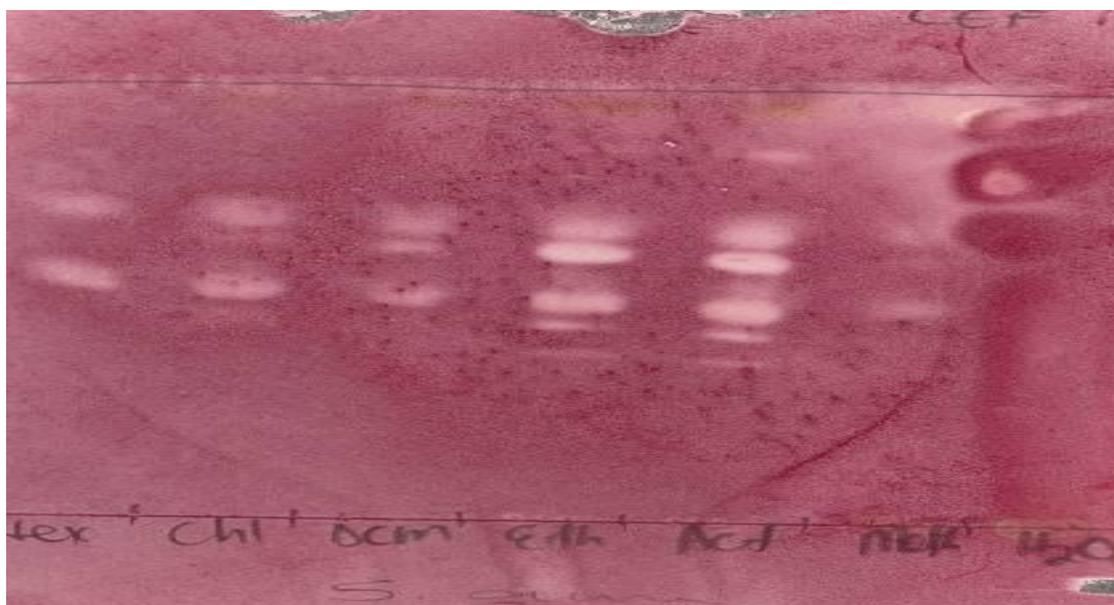


Figure 4: Bioautography of crude extracts developed in (CEF; 5:4:1) solvent system and sprayed with *staphylococcus aureus*.

Aldrich, South Africa) solution and further incubated for colour development. The clear zone against a red background indicates the inhibition of bacterial growth by bioactive compounds in the extracts.

Microplate dilution assay

Microplate dilution method was used to determine the minimum inhibitory concentration (MIC) values of the

extracts against each test bacterial species²⁷. The plant's extracts (100 µl) was serially diluted two-fold with water in a 96-well microplate, a 100 µl aliquot of test bacteria culture was added to each well. Acetone was used as a solvent control and distilled water was used as a negative control. Gentamicin was used as the positive control. The micro-plates were sealed and incubated for 24 h at 37 °C.

Table 5: MIC value of plant extracts against different bacteria.

Plant Extract	MIC values (mg/ml)			
	<i>S. aureus</i>	<i>E. coli</i>	<i>S. pneumoniae</i>	<i>B. subtilis</i>
Dichloromethane	0.63	0.63	0.31	0.63
Ethyl acetate	0.29	0.63	0.08	0.16
Acetone	0.16	0.16	0.04	0.16
Methanol	0.08	0.16	0.04	0.08
*Gentamicin	0.08	0.08	-	-

* = Standard

After incubation, 40 μ l of 0.2 mg/ml of mg/ml iodinitrotetrazolium (INT; Sigma-Aldrich, South Africa) solution was added to each well and the plates were incubated further for 2 h at 37 °C. The development of red colour, resulting from the formation of the red/ purple formazan, was an indication of microbial growth. The MIC values were regarded as the lowest concentrations of the extracts that inhibit the growth of the test organism. All tests were performed in triplicate and the antimicrobial activity was expressed as the mean of inhibition.

Statistical analysis

All the experiments were conducted in triplicate to ensure reproducibility. The data were presented as mean values \pm standard deviation ($n \pm SD$), $n = 3$.

RESULTS AND DISCUSSION

Quantity extracted

The quantities and percentage yields extracted from 3 g of the finely powdered leaves of *Rhus leptodictya* using different solvents are displayed in Figures 1. From the graphical data, it is evident that dichloromethane (DCM) had the highest percentage extractive potential compared to the other solvents used. The lowest extractive potential was observed with hexane, which showed about 3 % yield relative to the 35% observed with DCM. This, therefore, suggests that the leaves of *R. leptodictya* contain more polar compounds than non-polar compounds since more bioactive components are found in the methanol and DCM which is moderate and more polar respectively.

Thin layer chromatography fingerprinting

In this study, three mobile phase systems were used to separate the compounds based on their polarities and TLC fingerprinting are displayed in Figures 2. The mobile phase systems includes: chloroform/ethyl acetate/formic acid (CEF: 5:4:1); benzene/ethanol/ammonia hydroxide (BEA: 18:2:0.2); ethyl acetate/methanol/water (EMW: 40:5.4:5). The mobile phase: CEF showed good separation followed by EMW.

The developed TLC plates were sprayed with vanillin/H₂SO₄ solution after which it was heated at 100 °C to allow colour development. The identification of separated components was achieved on the basis of the retention factor (R_f) values (Table 1) and colours. Separated compounds visualization was achieved by natural colour in daylight or by fluorescent quenching on 254 nm (for conjugated double bonds or extended π electron systems) or 366 nm²⁸.

The chromatograms developed from different solvents systems showed a complex mixture of compounds confirmed by different colours due to reaction with

vanillin/H₂SO₄ spray reagent. Various class of compounds detected included terpenoids: purple or bluish purple colorations; flavonoids which are phenolic in nature (pinkish, yellow or orange); stilbenes (bright red to dark pink colour) and proanthocyanidins (pink colour)^{28, 29}. To confirm the presence of phenolic compounds, blue-black spots with ferric chloride-potassium ferric cyanide reagent were observed. The presence of flavonoids was confirmed by yellow spots when the spots reacted with aluminum chloride/acetic acid spray reagent (AlCl₃/CH₃COOH)^{28, 30}. Characterization of the phytochemical profile of the extracts indicated that the extraction method and the extractants used resulted in the complex mixture splitting displaying flavonoids as yellow colour intense in the ethyl acetate, acetone and methanol extracts.

Phytochemical constituents of *R. leptodictya* leave extracts

The results of phytochemical screening of *R. leptodictya* leaves as presented in Table 2 showed that the leaves are rich in phenols, tannins, saponins and flavonoids. However, the hexane and dichloromethane extracts showed the no presence of tannins. Phenolic compounds which are the largest and most abundant groups of plant metabolites were found in the leaves extracts of *R. leptodictya*⁵. The extract samples with phenolic and flavonoids compounds possess biological properties such as antiatherosclerosis, cardiovascular protection and improvement of endothelial function as well as inhibition of angiogenesis and cell proliferation activities^{4,31}. Phytochemicals such as saponins and tannins have anti-inflammatory effects³². Tannins are known to bind proline rich protein and interfere with protein synthesis, whilst on the other hand, saponins are known to produce an inhibitory effect on inflammation²⁸. They are also known to have the property of precipitating and coagulating red blood cells³³.

Total phenol and flavonoid contents of the extracts

Natural antioxidants from plants are normally found in the form of phenolic compounds such as flavonoids, phenolic acids, tocopherols etc. Several studies have shown that the higher antioxidant activity associated with medicinal plants is attributed to the total phenolic compounds³³. Flavonoids are natural antioxidants. They are well-known dietary biochemical agents, which show pH dependent antioxidant behaviour in the human body. In this study, the total phenolic content measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent in leaves of *R. leptodictya* extracts (standard curve equation: $y = 0.00048x + 0.0055$, $r^2 = 0.9873$) were between 0.003 \pm 0.001 mg/g and 0.38 \pm 0.002 mg/g (Table 3). In all, methanol extract (0.38 \pm 0.002 mg/g) and acetone extract

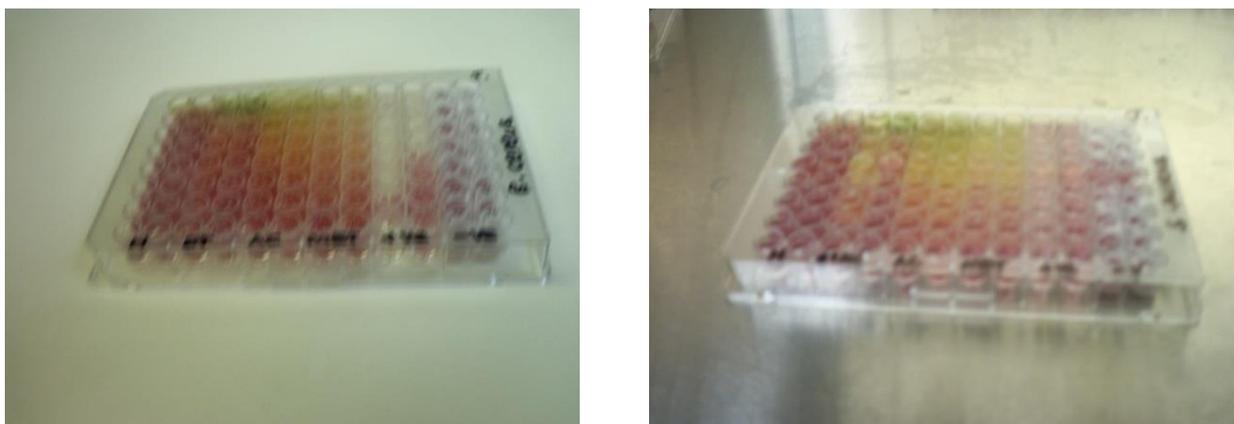


Figure 5: 96 well MIC plates of extracts with bacteria and INT after 12 h.

(0.21 ± 0.007 mg/g) exhibited the higher values of phenol contents than other extracts. Table 3 equally show the flavonoid contents of leaves of *R. leptodictya* in the hexane, dichloromethane, acetone, ethyl acetate and methanol extracts in terms of quercetin equivalent (the standard curve equation: $y = 0.0092x + 0.0249$, $r^2 = 0.985$). The total flavonoid varied from 2.35 ± 0.2 to 18.22 ± 2.1 mg/g of the extract powder. Acetone extract with total flavonoid contents of 18.22 ± 2.1 mg/g exhibited the highest amount amongst the studied extracts while hexane had the lowest contents (2.35 ± 0.2 mg/g).

Phenols are important mainly because of their function to scavenge the free radicals in the human body and to help maintain a healthy body by scavenging or removing the reactive oxygen species (ROS)¹⁸. The results revealed that the leaves of *R. leptodictya* are rich in phenols. The potential beneficial effects of the high antioxidant activity and protections of cells from free radical attack seem clear¹⁷. This confirms the assertion that phenolic content of plants contributes directly to their antioxidant properties. The values recorded for all extracts showed that the leaves of *R. leptodictya* are a relatively good source of antioxidant activity. Flavonoids are hydroxylated phenolic substances which are synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against a wide range of microorganisms. The activity is due to their ability to complex with extracellular and soluble proteins and to form a complex with bacterial cell wall^{16,17}. They are also known to be effective antioxidant and show strong anticancer activities³². These molecules are also effective for the cardiovascular system and work as cardio protective agents. The high contents of these phytochemicals in *R. leptodictya* plant extract also explain its high radical scavenging activity.

Antioxidant activity

The therapeutic benefit of medicinal plants is often attributed to their antioxidant property. Antioxidants play very important roles in coetaneous tissue repair as they significantly prevent tissue damage that stimulates wound healing process^{16,18}. The antioxidant capacity of the leaves of *R. leptodictya* was used to determine the active extracts as measured by the TLC-DPPH method. The TLC chromatograms are presented in Figure 3.

The following solvent systems of varying polarity: Hexane: Ethyl acetate in (95:5), (90:10), (85:15) and (80:20) ratios were prepared to run the TLC for detecting antioxidant activity in the leaves extracts. Developed chromatograms were sprayed with 0.2 % 2,2-diphenyl-2-picrylhydrazyl (DPPH) in methanol, as an indicator. In the chromatograms eluted with Hexane: Ethyl acetate (80:20), there is some activity at the top of the plate of the polar fractions. The polar extractants, methanol, and acetone showed the best activities in all solvents systems compared to other extracts. This is due to the polarity of the components of the compounds present in the extractant and moreover, most antioxidant constituents are polar secondary metabolites phenolic compounds especially flavonoids^{4,5}. The presence of antioxidant compounds was detected by yellow spots against a purple background on TLC plates. The areas of inhibition (coloured yellow) were compared with the R_f value of the related spots on the reference TLC plate. The presence of clear bands on the plates against the purple background (Figure 3) indicated growth inhibition²⁵. From these TLC chromatograms, it is clear that the methanol, acetone, and ethyl acetate fractions contained more antioxidant compounds than other fractions; however, the number of constituents with antioxidant activity varied amongst the studied solvent extracts. Masoko and Eloff³⁴ observed that acetone and methanol extracts exhibited the presence of antioxidant activity after spraying the TLC chromatogram with DPPH. The quantitative antioxidant activity of hexane, dichloromethane, acetone, ethyl acetate and methanol extracts of *R. leptodictya* were also determined spectrophotometrically by DPPH assay using ascorbic acid as standard as given in Table 4.

Among the extracts studied, acetone showed maximum antioxidant capacity ($IC_{50} = 0.134 \pm 0.018$ mg/ml) whereas the antioxidant activity (IC_{50}) exhibited by methanol and ethyl acetate were 0.212 ± 0.076 and 0.328 ± 0.04 mg/ml respectively (Table 4). These results revealed that acetone possesses more extractive efficiency followed by methanol than other solvents. Although, the activities exhibited by the plant extracts in this study were low when compared to that of the standard agent: Ascorbic acid ($IC_{50} = 0.125 \pm 0.013$ mg/ml). The DPPH radical scavenging ability of the extracts can be ranked as Acetone > Methanol > Ethyl

acetate > Dichloromethane > Hexane. Similar promising results have also been reported for methanol and acetone extracts of plant species^{21,25,26}.

TLC Bioautography assay

The bioautography assay was used to determine the antibacterial activity of active compounds present in leaf extracts of *R. leptodictya* on the surface of chromatographic plates and layering the TLC plate with molten bacterial agar. The clear zones indicate the inhibition of bacterial growth by bioactive compounds in the extracts as observed on the TLC plates (Figure 4) as spots on red/ pink background when sprayed with an aqueous solution of *p*-iodonitrotetrazolium violet (INT).

Acetone and ethyl acetate fractions showed the highest number of antibacterial active compounds against *Staphylococcus aureus* followed by the crude extract of the dichloromethane. The observed spots with R_f value ranging from 0.68-0.87 correspond to the spots representing flavonoids on spraying the TLC plate with 1 % ethanolic solution of aluminum chloride. Hexane, methanol showed only two spots which are active. The results obtained in this assay showed that the leaf extracts of *R. leptodictya* possess good antibacterial activity against *S. aureus* in the acetone and ethyl acetate fractions due to the presence of flavonoids⁵. The observed inhibition by these fractions might due to overlapping of one or more bioactive components possibly due to the solvent system. The result obtained in this study is in agreement with the antibacterial efficacy of flavonoids and tannins against *S. aureus* as reported by Soni and Dahiya³⁵.

Minimum Inhibitory Concentrations (MIC) activity

The antimicrobial activity of the dichloromethane, acetone, ethyl acetate and methanol extracts of *R. leptodictya* leave was studied using four bacterial isolates by microplate dilution method. Although, the extracts exhibited moderate inhibition against the studied bacterial, methanol and acetone extracts were found to have potent antimicrobial activity against the Gram-positive and Gram-negative bacteria (Table 5). This suggests that these fractions have a broad spectrum of antimicrobial activity. Gram-negative bacteria have been known to show resistance to antimicrobial agents due to the composition of their cell membrane^{1,2}. Plant extracts with low MIC values could be a good source of bioactive compounds with antimicrobial strength³⁶. The methanol and acetone fractions had the lowest MIC value of 0.04 mg/ml against *S. pneumoniae*, while methanol extract possesses 0.08 mg/ml against *S. aureus* and *B. subtilis*. The ethyl acetate and dichloromethane fractions investigated showed varying results as against the bacterial, with ethyl acetate extract possessing 0.08 and 0.29 mg/ml against *S. pneumoniae* and *S. aureus* (Gram-positive) respectively. High MIC value was found in ethyl acetate and dichloromethane fractions (0.63 mg/ml) against *E. coli*; a Gram-negative bacteria. Amongst the fractions, only methanol extracts exhibited antibacterial activity (0.08 mg/ml) comparable to that of the standard agents: Gentamicin against *S. aureus*. Dichloromethane extract was observed to be less active against *B. subtilis*, *E. coli* and *S. aureus* when compared to the acetone and methanol

extracts (Table 5). It is, however, active against *S. pneumoniae* since its MIC is about 0.31 mg/ml. The bacterial growth in the microtitre plates was indicated and detected with the use of a bacterial growth indicator, *p*-iodonitrotetrazolium violet (INT). The INT indicates the presence of viable bacteria in the microtitre plate suspensions by acting as an electron acceptor for the bacteria to form a red-coloured formazan product^{26, 36}. After INT addition and incubation of the microtitre plates, the presence of antibacterial activity could be assessed with the naked eye. Antibacterial activity was indicated by the absence of the red-coloured formazan³⁶ (Figure 5). Table 4 gives a clear indication that all the extracts were very active against *S. pneumoniae*.

CONCLUSION

It is interesting to note that the integral part of nature is herbal plants which contain natural substance essential to promote health. From this study, acetone and methanol extracts were found to be the best solvents for the phytochemical screening of *R. leptodictya* leaves. The extracts were found to contain a variety of compounds, most of which were flavonoids and phenols, possessing antibacterial activity against *S. aureus*, *S. pneumoniae* and *B. subtilis*. The bioautography assay showed that the leaf extracts of *R. leptodictya* possess good antibacterial activity against *S. aureus*. Acetone extract showed maximum radical scavenging capacity (0.134 ± 0.018 mg/ml). The acetone and methanol extracts showed a high number of bioactive compounds in the preliminary phytochemical assay and a good amount of total phenol and flavonoid contents in the antioxidant and antibacterial activities, which could be used in food and therapeutic applications. Since many flavonoids possess both antimicrobial and antioxidant activities as well as a nutritional supplement; this may mean that there are synergistic antioxidant activities between the different components of the crude extracts. Hence, isolation of the bioactive components would be of interest for further studies.

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