Phytochemical Screening, GCxGC TOF-MS Analysis and Antibacterial Properties of Crude *Rhoicissus Ttomentosa* Rhizome Extract

Nkemdinma Uche-Okereafor, Derek Ndinteh, Nicolette Niemann, Vuyo Mavumengwana

Abstract*— *Rhoicissus tomentosa is a plant used in South African traditional medicine to treat ailments mainly related to fertility and reproduction. Phytochemical screening was carried out and the antimicrobial activity of the rhizomes of *R. tomentosa* was investigated for the first time to ascertain their possible pharmaceutical potential. The results showed that the rhizomes of the plant contain alkaloids, flavonoids, saponins, steroids, reducing sugars and tannins. GCxGC-TOF-MS analysis of the extracts displayed the presence of over 100 known bioactive compounds, many reported here and detected in *Rhoicissus tomentosa* for the first time. Methanol: chloroform (50/50, v/v) and ethyl acetate (100%) extracts of the rhizomes were tested against 14 bacterial strains using the disc diffusion and microdilution assay methods. Bacteria most susceptible to rhizome extracts were *Staphylococcus aureus* (0.063 mg/mL) and *Bacillus subtilis* (0.125 mg/mL). The results obtained show that the rhizomes of *R. tomentosa* have antimicrobial activity against a number of pathogenic microorganisms.

***Index Terms*—** Disc diffusion**,** GCxGC TOF-MS microdilution, Phytochemical screening, *Rhoicissus tomentosa*.

I. Introduction

Antibiotic resistance is a form of drug resistance whereby some sub-populations of microorganisms, usually bacterial species, are able to survive after exposure to one or more antibiotics [1]. The use of plant extracts for therapeutic purposes became popular when people realized some of the drawbacks (bacterial resistance, over prescription and misuse) associated with antibiotics [2, 3]. In recent times, medicinal plants are finding their way into many pharmaceuticals, cosmetics and neutraceuticals. Some of these medicinal plants have given Western pharmacopoeia about 7000 different pharmacologically important compounds and a number of top selling drugs of modern times [3]. Despite past discoveries from a number of many important plants with pharmaceutical properties, still many plant species have not been phytochemically characterized and remain unknown to science. Thus, it is reasonable to expect that new plant sources of valuable and pharmaceutical interest remain to be discovered and developed [4].

Rhoicissus tomentosa is a medicinal plant indigenous to Southern Africa [5, 6]. It is known as "Wild Grape" in English, "idiliya" in Xhosa and "isiNwazi" in Zulu [7]. Traditionally, the boiled roots mixed with other plants are used to enhance fertility [8], the roots boiled in milk are administered as anthelmintic to calves and the stem and leaves are used during pregnancy to ensure a safe delivery [9].

In this paper, the antimicrobial activities, phytochemical screening and two dimensional gas chromatography coupled with time of flight mass spectrophotometry (GCxGC-TOF-MS) analysis of crude extracts of *R. tomentosa* were investigated to ascertain its usefulness as a possible drug lead. Chemical analysis of a crude extract using the GCxGC-TOF-MS were used to characterize volatile compounds in the plant.

II. Procedure

A. Sample Collection and Preparation

Plant material (rhizomes of *R. tomentosa*; 3kg) was purchased from the Faraday Muthi market in Johannesburg in February 2015. A herbarium sample was submitted to the University of Johannesburg Herbarium with reference number BTNNU01. The collected plant material was cut into smaller pieces and dried at 40 ˚C in an oven for five days.

The method described by [10] with slight adjustments was used for the preparation of the crude extracts.

B. Phytochemical screening and analysis

Phytochemical tests of the powdered bark were carried out by the methods outlined by [11] and modified by [12].

GCxGC-MS-TOF is a powerful multidimensional Gas Chromatography technique that combines two independent separations to accurately analyze highly complex samples. All reagents used in this analysis were of analytical grade and were purchased from Sigma Aldrich. The crude ethyl acetate extract of *R. tomentosa* was analyzed. The extract was first

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N.C. Uche-Okereafor is with the *Department of Biotechnology and Food Technology, University of Johannesburg, Johannesburg, South Africa*

N. Niemann is with the *Department of Biotechnology and Food Technology, University of Johannesburg, Johannesburg, South Africa*

V. Mavumengwana is with the *Department of Biotechnology and Food Technology, University of Johannesburg, Johannesburg, South Africa*

D.T. Ndinteh is with the *Department of Applied Chemistry, University of Johannesburg, Johannesburg, South Africa*

homogenized by weighing 0.5 g into a vial then adding 200 µl of cold methanol. The mixture was thoroughly vortexed. The sample was transferred to an auto sampler vial and immediately analyzed. The sample was analyzed under the instrumental conditions outlined by [13].

C. Antibacterial screening

Crude extracts of *R. tomentosa* were tested against 14 specific bacteria strains: *Bacillus cereus (ATCC10876), Bacillus subtilis (ATCC19659), Enterobacter aerogenes* (*ATTC13048*), *Enterobacter cloacae (ATCC13047)* , *Enterococcus faecalis (ATCC13047), Escherichia coli (ATCC25922), Klebsiella oxytoca (ATCC8724), Klebsiella pneumonia (ATCC13882), Mycobacterium smegmatis (MC^2 155), Proteus mirabilis (ATCC7002), Proteus vulgaris (ATCC6380), Pseudomonas aeruginosa (ATCC27853), Staphylococcus aureus (ATCC25923), Staphylococcus epidermidis (ATCC14990)*. Agar disc diffusion was carried out according to the method described by [14] and the experiment was done with five repetitions. Minimum Inhibitory Concentrations were carried out according to the method outlined by [15] and the experiment was done in five repeats using 96-well micro titer plate.

III. Results and Discussions

Phytochemical screening

Phytochemical screening of *R tomentosa* was performed according to the methods mentioned and results obtained are displayed in Table 1. The rhizomes tested negative for cardiac glycosides but positive for tannins, flavonoids, steroids, reducing sugars, alkaloids and saponins.

TABLE I
PHYTOCHEMICAL SCREENING OF *R. TOMENTOSA*

Phytoconstituents	Observation	Results
Tannins	Blackish-blue/ Blackish-green coloration.	Positive
Flavonoids	Yellow coloration	Positive
Steroids	Reddish brown ring	Positive
Reducing sugars	Bright blue	Positive
Alkaloids	Turbidity/ Precipitation	Positive
Saponins	Persistent froth	Positive
Cardiac glycosides	No brown ring at interface	Negative

Disc diffusion

Different solvents were used to extract compounds from *R. tomentosa* rhizomes and both extracts were tested for antibacterial properties. The results from the disc diffusion experiments are shown in Table 2. The methanol/chloroform (50:50 v/v) crude extract showed significant zones of inhibition on the agar plates against *B. cereus, E. faecalis, K. pneumoniae, M. smegmatis, P. mirabilis* and *P. vulgaris*. The 100% ethyl acetate extract showed zones of inhibition against the same bacterial strains as the methanol/chloroform extract and also against *S. epidermidis.* When compared to streptomycin, the extracts had noteworthy zones of inhibition since they did not contain a single pure compound but a mixture of chemicals. On average the 100 % ethyl acetate extract showed better zones of inhibition.

TABLE II
DISC DIFFUSION OF THE CRUDE EXTRACT ON DIFFERENT BACTERIAL STRAINS

Test organism	Ext 1 zones of inhibition (in mm)	Ext 2 zones of inhibition (in mm)	Control zones of inhibition (in mm)
B. cereus	10	11	28
B. subtilis	NI	NI	20
E. faecalis	7	11	20
M. smegmatis	10	11	23
S. aureus	NI	NI	25
S. epidermidis	NI	9	25
E. aerogenes	NI	NI	21
E. cloacae	NI	NI	20
E. coli	NI	NI	25
K. oxytoca	NI	NI	24
K. pneumoniae	8	8	29
P. mirabilis	11	10	24
P. vulgaris	13	14	27
P. aeruginosa	NI	NI	24

NI = No Inhibition, Ext. 1 = 50:50 Methanol/Chloroform extract, Ext 2 = 100% Ethyl acetate extract, Control = Streptomycin

Minimum Inhibitory Concentration (Microdilution method)

The Minimum Inhibitory Concentration (MIC) results showed that the extracts from *R. tomentosa* rhizomes were very antibacterial against *Bacillus cereus* (MIC at 0.500 mg/mL) and *B. subtilis* (MIC at 0.125 mg/mL). *Staphylococcus aureus* was also very sensitive to the extracts with a MIC of 0.063 mg/mL. The ethyl acetate extract showed better antibacterial properties against a number of bacteria, most notably *Mycobacterium smegmatis* (MIC 0.063 mg/mL), *Enterococcus faecalis* (MIC 2.000 mg/mL) and *Staphylococcus epidermidis* (MIC 2.000 mg/mL).

TABLE III
MIC OF THE CRUDE EXTRACT ON DIFFERENT BACTERIAL STRAINS

Test organism	Ext 1 (MIC mg/mL)	Ext 2 (MIC mg/mL)
B. cereus	**0.500**	**0.500**
B. subtilis	**0.125**	**0.125**
E. cloacae	>16.000	>16.000
E. aerogenes	>16.000	>16.000
E. faecalis	>16.000	**2.000**
E. coli	>16.000	>16.000
K. oxytoca	>16.000	>16.000
K. pneumoniae	>16.000	>16.000
M. smegmatis	>16.000	**0.063**
P. mirabilis	**16.000**	>16.000
P. vulgaris	**16.000**	**8.000**
P. aeruginosa	>16.000	>16.000
S. aureus	**0.063**	**0.063**
S. epidermidis	>16.000	**2.000**

Ext. 1 = 50:50 Methanol/Chloroform extract, Ext 2 = 100% Ethyl acetate extract,

GCxGC-TOF-MS

GCxGC-TOF-MS analysis was done to determine the

chemical make-up of the plant material and analyze the volatile components. The result showed prevalent presence of many bioactive chemicals of both known and unknown components. In Table 4 are some of the volatile chemicals that were identified by using the ChromaTOF® software and a NIST database.

The tables below, grouped according to the nature of compounds, contains a list of the volatile chemicals present at highest concentrations in the crude rhizome extract. Many fatty acids and amino acids are present on this list, and they are usually the building blocks for cell membranes and proteins (enzymes). Yet many of these compounds have also been found to have antimicrobial effects, so they may contribute significantly to the antibacterial properties of the plant.

Tables 4-6: Showing compounds identified in *R. tomentosa* by GCxGC-TOF-MS at highest concentrations and the known bioactivity of the compounds.

TABLE IV
TABLE SHOWING THE AMINO ACIDS IDENTIFIED FROM R. TOMENTOSA RHIZOMES USING GCXGC-TOF-MS

Name of Compound	Area %	Biological activity
D-Asparagine	0.025	Immunostimulant, antibacterial, anti-infective & analgesic activities [16].
L-Arginine	0.002	Anti-inflammatory, Immunostimulant & antihypertensive activities [17, 18].
Glycyl-L-valine	0.037	Analgesic, antipyretic, antioxidant & anti-inflammatory activities [19].
Uridine	0.033	Neuroprotective activity, pyrimidine metabolism, antidepressants and anti-epileptic actions [20, 21, 22, 23].

TABLE V
TABLE SHOWING THE CARBOXYLIC ACIDS IDENTIFIED FROM R. TOMENTOSA RHIZOMES USING GCXGC-TOF-MS

Name of Compound	Area %	Biological activity
Hexadecanoic acid	2.785	Antioxidant, antibacterial, anthelminthic and antifungal activities [24, 25].
Hexadecanoic acid, methyl ester	0.016	Anti-spasmodial, antioxidants, anti-abortifacient activities [26, 27, 28, 29, 30].

TABLE VI
TABLE SHOWING THE FATTY ACIDS IDENTIFIED FROM R. TOMENTOSA RHIZOMES USING GCXGC-TOF-MS

Name of Compound	Area %	Biological activity
cis-9-Octadecenoic acid	27.296	Antioxidant, anti-inflammatory, anti-tumor, anti-spasmodial & antimicrobial activities [31, 32, 33].
Eicosanoic acid	0.121	Anti-abortifacient, antioxidant, antibacterial, analgesic, antipyretic activities [34, 35].
Docosanoic acid	0.238	Antipruritic, antioxidant, anesthetic activities [36].
Decanoic acid, 2-propenyl ester	0.013	Analgesic, antipyretic, antibacterial, antifungal & anti-inflammatory activities [37].
Octadecanoic acid	0.236	Antifungal, anti-tumor, antibacterial and antioxidant activities [35, 38].
Tetradecanoic acid	0.448	Antipruritic, antifungal, anti-infective & antioxidant activities [39, 40, 41].
Tetracosanoic acid	0.041	Antibacterial activity [42].
9-Octadecynoic acid	0.004	Antifungal, cytotoxicity, anti-asthmatics, antidepressants activities [43].

Medicinal plants are rich sources of bioactive chemicals and these compounds have been known to possess beneficial properties to human health in addition to the nutritional benefits they may have [44].

The results of the phytochemical screening carried out on the rhizomes of *R. tomentosa* revealed the presence of many known groups of bioactive compounds: tannins, flavonoids, steroids, reducing sugars, alkaloids and saponins.

Tannins are polyphenolic compounds which possess anti-inflammatory, antiseptic and antioxidant properties [45]. In Chinese and Japanese natural healing, most tannin-containing plants are used as astringents against diarrhoea, as diuretics and against stomach duodenal tumors [46]. Flavonoids are also polyphenolic compounds and have been reported to have multiple biological activities which include antimicrobial, cytotoxicity, anti-inflammatory, anti-tumor, anti-allergy, vascular- and oestrogenic activities. Flavonoids' most important bioactivity is that they are powerful antioxidants thus allowing them to scavenge free radicals and protect the body from reactive oxygen species [47, 48].

Steroids have been reported to possess antibacterial properties [49] and they are very important compounds especially due to their relationship with sex hormones [50].

Alkaloids have a variety of biological activities including cytotoxicity which is the most common [51]. Its other biological activities may be analgesic [52, 53], antispasmodic, antibacterial [54, 55], antihypertensive, antimalarial and anti-cancer [56].

Saponins are mostly characterized by their ability to foam in aqueous solutions. Some of its bioactivity include haemolytic activity and cholesterol binding property [57,58], antimicrobial, anti-protozoan, immunostimulant, anti-carcinogenic and antioxidant activities [59, 60, 61].

Reducing sugars act as anti-diabetics by reducing blood glucose levels [62].

The phytochemical results for the presence of flavonoids and saponins support previous findings [5], but the other phytoconstituents to the best of our knowledge are being reported present in the *R. tomentosa* rhizomes for the first time.

The antibacterial screening results showed that the disc diffusion and Minimum Inhibitory Concentration gave slightly different results. The two different extracts also showed varying results when compared to each other. For the methanol/chloroform extract, disc diffusion test results showed that growth in *B. subtilis* and *S. aureus* were not inhibited but in the MIC test they were inhibited. The MIC test on the other hand have showed no inhibitory activity against *E. faecalis, M. smegmatis* and *K. pneumonia* but their growth were inhibited during the disc diffusion test. The MIC results and disc diffusion results for the ethyl acetate extract were similar except that in the disc diffusion test it did not show activity against *B. subtilis* and *S. aureus* but in the MIC test it could inhibit these two species and the MIC test showed no inhibitory activity against *K. pneumonia* and *P. mirabilis* but the disc diffusion test showed inhibition from this extract.

Of the 14 bacterial strains screened for antibacterial activity, 8 were susceptible to the crude extracts of *R. tomentosa* at the starting concentration (16 mg/mL) and below while the rest did not display sensitivity at the concentrations tested. It is still possible for those bacteria strains to be susceptible to the plant material at higher concentrations.

The disc diffusion test is not a very accurate method for testing bacterial susceptibility because the sample impregnated on the disc might not diffuse properly into the agar, hence there may develop no zone of inhibition. Also it can only tell if samples are active against particular bacterial strains but will not give the concentrations at which the bacteria are susceptible.

The antibacterial screening results of the rhizomes of *R. tomentosa* corresponds with previous findings by [9] on the antibacterial activity of the leaves and stem of *R. tomentosa*. This is the first report on the antibacterial activity of the rhizomes of *R. tomentosa*. From the results, we observed that the crude extracts of *R. tomentosa* showed better antibacterial activity against the Gram-positive organisms: *B. cereus, B. subtilis, E. faecalis, M. smegmatis, S. aureus* and *S. epidermidis* than Gram-negative ones: only *P. vulgaris* and *P. mirabilis* displayed sensitivity to the extracts.

The GCxGC TOF-MS results revealed a number of biologically active chemicals which might contribute to the plant's antibacterial activity and its uses in traditional medicine, however, the relationship between these phytoconstituents and the biological activities and ethnobotanical uses of *R. tomentosa* have not been established. Garlic acid for example (Trihydroxybenzoic acid) contained in *R. tridentate* [63] was also identified in *R. tomentosa* using GCxGC TOF-MS analysis. There is a possibility that other chemicals contained in *R. tomentosa* may still be found in *R. tridentate* and possibly other *Rhoicissus* species hence they could possibly be used to treat similar disease symptoms. This validates the studies of [8]. This is the first report of GCxGC-TOF-MS analysis on *R. tomentosa* rhizomes and the presence of many compounds in the rhizomes are reported for the first time.

IV. CONCLUSIONS

R. tomentosa rhizomes showed that at a low concentrations of 0.5mg/mL and 0.125 mg/mL respectively there was inhibitory activity against *B. cereus* and *B. subtilis*; at 0.0625 mg/mL it showed impressive inhibition of *S. aureus* and *M. smegmatis*. With MIC of 2 mg/mL it showed activity against *E. faecalis*, and *S. epidermidis* too which suggests that the plant might be a potentially good source of raw material for possible antimicrobial medications. The ethyl acetate extracts showed heightened bioactivities against more bacteria strains than the methanol and chloroform extract which also suggests that ethyl acetate as a solvent most probably extracted some good bioactive compounds which were not extracted by the methanol and chloroform. The phytochemical screening and GCxGC-TOF-MS analysis gave an insight on the general chemical components of the plant. The results of the study gives credence to the traditional use of this plant to treat diseases.

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Uche-Okereafor Nkemdinma Chinezurum a 27 year is a Nigerian citizen. She obtained a BTech degree in Industrial Microbiology from the Federal University of Technology, Owerri, Nigeria in 2012. She is currently studying towards an award of a Master's degree (MSc) in Biotechnology at the Department of Biotechnology and Food Technology, Faculty of Science, University of Johannesburg (UJ), South Africa with research focus on plant natural products and drug discovery.

She actively participated in the 2015 University of Johannesburg Cross Faculty symposium via a poster presentation, the 2015 Howard University America/Department of Science and Technology, South Africa Women in Science Technology, Engineering and Mathematics (STEM) Conference held at Rosebank, Johannesburg, South Africa, via a podium presentation and the 2016 Autumn International Scientific Conference on Food Safety and Security, Johannesburg, South Africa, via a poster presentation.

Mrs Uche-Okereafor aims to strive well and reach the highest academic ladder where she will be able to to pursue her career in academics and research with special emphasis in drug discovery and development from medicinal plants.