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Characterisation of secondary metabolites from *Rhinacanthus nasutus* L (Kurz) for the identification of novel antibacterial leads

Sheikh, H.M.[1,2*] and Reshi, N.A.[3]
[1]Department of Biological Science, Faculty of Science and Arts, King Abdulaziz University, Rabigh Campus, P.O. Box 344, 21911 Rabigh, Saudi Arabia
[2]Biology Department, Faculty of Science, University of Jeddah, P.O. Box 80327, Jeddah, 21589, Saudi Arabia
[3]Department of Botany, School of Science, Sandip University, Nashik, Maharashtra, 422 213, India
[*]Corresponding author e-mail: hmsheikh@kau.edu.sa
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Abstract. The bioactivity of *R. nasutus* leaf extracts was assessed on *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Vibrio parahaemolyticus*, *Enterobacter aerogenes*, *Proteus mirabilis*, and *Klebsiella pneumoniae.* Crude chloroform, petroleum ether, ethyl acetate, ethanol and methanol extracts were screened by disc diffusion method. Promising crude extract was further subjected to the column fractionation followed by the screening of the antibacterial activity of individual fractions. Biologically active pure fraction was subjected to the advanced analytical studies like HPLC, LC-MS, IR and NMR for characterisation of the bioactive compound. Ethanolic extract exhibited the maximum antibacterial activity against *Klebsiella pneumoniae* with the maximum of 35±0.42 mm zone of inhibition. The biologically potent column fraction from ethanol extract with 40±0.42 mm zone of inhibition upon subject to the HPLC, LC-MS, IR and NMR revealed that the active compound is rhinacanthin-C, a naphthoquinone.

INTRODUCTION

Ever since the existence of life there has been an endless encounter between humans and the infectious microorganisms. Major advancement in the antibiotic developments in the 20th century helped turn the tide in favor of humans and with the discovery of penicillin in early 1940s the situation drastically improved with respect to the bacterial diseases (Magiorakos *et al.*, 2012). However, the ecstasy over potential defeat of infectious disease had a short existence. Almost as soon as antibacterial drugs were deployed, bacteria started developing various forms of resistance against the available drugs. With the increased usage of antibacterials, the bacterial pathogens exhibited higher levels of complexity of resistance against the available antibiotics (Shah *et al.*, 2007). There has been a continuous struggle to gain the upper hand against deadly infectious microorganisms and despite the advancements in antibiotic research in the recent past, development of drug resistance is a grave and grim threat due to the scarcity in the contemporary drug development pipeline which are effective against vancomycin-resistant *Klebsiella pneumoniae*, multidrug resistant (MDR) *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), β-lactamase producing Gram-negative bacteria or MDR and extensive-drug resistant (XDR) strains of *Mycobacterium tuberculosis* (Pillay *et al.*, 2007). The development of resistance is attributed to the widespread overuse and over-the-counter (OTC) availability of antibiotics. The present situation is fostering prospective research

to identify the new biologically viable natural molecules from the traditional medicine.

The increasing prevalence of drug resistant bacteria has pressed hard on scientific communities to look for potential antibacterial agents of plant origin, which are considered as untapped diverse source of chemicals. Plants are considered as treasure house of biologically active principles which makes them viable source of different remedies (Tadeg *et al.*, 2005). Contrary to the synthetic drugs, antimicrobials of plant origin are devoid of any side effects and have tremendous curative potential to heal infectious diseases. Many attempts have been made to unfold the new antimicrobials from different natural resources such as microorganisms, animals and plants. Till date, approximately 20% of the medicinal plants worldwide have been explored to identify and unravel their biologically active compounds (Sukanya *et al.*, 2009). Bioprospecting of traditionally used medicinal plants forms the basis of drug discovery.

Rhinacanthus nasutus belongs to the family Acanthaceae (shrub, 1-2 cm in height) and is extensively used in the traditional medicine of India. The plant grows in India, Taiwan, Thailand, and South Africa. Leaves and roots of this plant are extensively used for the treatment of various diseases (Wu *et al.*, 1988). Traditionally, it is used to treat cancer, rheumatism (Kupradinun *et al.*, 2009), pulmonary tuberculosis, peptic ulcers and scurvy (Sendl *et al.*, 1996), antimicrobial activity (Puttarak *et al.*, 2010), antiantidiabetic activity (Upendra *et al.*, 2010).

MATERIALS AND METHODS

R. nasutus plants were collected from the Nilgiri Hills, Western Ghats of Tamil Nadu, India and are being maintained in the medicinal plant garden of Department of Botany, Sandip University, Nashik, India. Leaves were shade dried and coarse powdered using electric homogeniser. Solvent extraction of plant material was carried out by taking 25 grams of dry leaf coarse powder in a thimble and extracted sequentially with 200 ml of petroleum ether, chloroform, ethyl acetate, ethanol and methanol in Soxhlet extractor for 48 hours (Hawthorne *et al.*, 2000). The solvent extracts were concentrated under reduced pressure and were stored at 5°C in vials for further use. The test bacteria were; **Gram-positive bacteria:** *Streptococcus pyogenes* (MTCC 422), *Staphylococcus aureus* (MTCC 1144), *Bacillus subtilis* (MTCC 441), *Bacillus cereus* (MTCC 430). **Gram-negative bacteria:** *Klebsiella pneumoniae* (MTCC 7407), *Proteus mirabilis* (ATCC 7002), *Enterobacter aerogenes* (MTCC 111) and *Escherichia coli* (MTCC 1687). All the test microorganisms were procured from MTCC Chandigarh, India. The preliminary antibacterial activity was screened by Disc Diffusion Method (Drew *et al.*, 1972). The extract showing the promising antibacterial activity was subjected to the column fractionation using methanol and 5% aqueous acetic acid (80:20, v/v) as the mobile phase (Ignat *et al.*, 2011). The individual fractions were further confirmed on TLC followed by screening of antibacterial activity of pure fractions by disc diffusion method (Drew *et al.*, 1972). The pure fraction with the maximum inhibition potential was subjected to the HPLC, LCMS, IR and NMR for the structural elucidation. **HPLC:** Symmetric shield RP18 (150 mm x 3.9 mm x 5 µm) column was used. 10 mM ammonium acetate and acetonitrile were used as mobile phase with the flow rate of 1 ml/min. 2 mg of extract sample was dissolved in DMSO and was injected into column at the volume of 10 µl and the column was run for 12 minutes. The sample analysis was carried out at room temperature and the chromatogram developed was used for further studies to elucidate the compound structure.

LC-MS: It was done using SPD 10 AVP – Shimdzu apparatus with column of phenomenex RP 18 of 25 x 2.5 dimensions. The mobile phase used was water: methanol (1:1), 20 µl sample was injected with the flow rate of 2 ml/min. The LC was detected at 265 nm using photo diode array detector. **IR:** The pin head size dry sample was placed on the detector and the IR was recorded. The

main functional groups were identified by their characteristic vibrational frequencies (Kalsi, 2002). IR spectrum was recorded using PerkinElmer Spectrum IR spectrometer in the range of 4000–600 cm^{-1}. Two types of NMR studies were carried out; Proton NMR (^{1}H-NMR) and Carbon NMR (^{13}C-NMR). NMR spectrometer of Bruker DR x 500 FT- NMR was used for analysis. The sample was dissolved in DMSO and was injected.

RESULT AND DISCUSSION

The zone of inhibition was measured in mm after 24 hours of incubation and the results revealed that all the leaf extracts inhibited the growth of both Gram negative and Gram positive bacteria. The zone of inhibition of different crude extracts is shown in Table 1. The maximum zone of inhibition was reported in ethanol extract against *Klebsiella pneumoniae* with 35±0.42 mm zone of inhibition. Ethanol crude extract upon column fractionation resulted in 4 elutes and each fraction was subjected to the TLC. Out of the four elutes tested for antibacterial activity, fraction 2 exhibited the maximum zone of inhibition of 40±0.42 mm (Table 2). The HPLC chromatogram of the active fraction showed two peaks (a major and a minor) and the peak which eluted at 3.32 min is predominate with the maximum absorbance at 200 nm (Fig. 1). The LCMS analysis reveals a major peak with a mass of 410.5026 (Fig. 2). The IR spectra reveal a broad and strong band at 3375.73 cm^{-1} indicating the presence of -OH group. The stretching vibrations are due to the presence of aliphatic and aromatic -OH groups at signals 2295.21 cm^{-1} and 2924.35 cm^{-1}. The stretching vibrations of aromatic rings at 1545.12 cm^{-1} suggest the presence of C=C. The presence of ester carbonyl group is indicated by intense absorption at 1726

Table 1. Inhibition Zone (mm) of leaf extracts against different Gram positive and Gram negative bacteria

Pathogen	Ethanol	Methanol	E. Acetate	Pt. Ether	Chloroform	Streptomycin
B. cereus	19±0.63	–	–	4±0.54	–	40±0.55
B. subtilis	22±0.65	15±0.34	5±0.45	–	2±0.54	35±0.65
S. pyogenes	–	–	–	–	–	38±0.43
S. aureus	27±0.65	5±0.45	12±0.45	2±0.33	3±0.58	35±0.23
E. coli	14±0.23	2±0.65	–	–	–	40±0.53
P. mirabilis	15±0.28	12±0.67	3±0.62	–	–	38±0.54
K. pneumoniae	35±0.42	–	–	–	–	35±0.56
E. aerogenes	15±0.28	4±0.53	–	–	–	35±0.76

* Each test was repeated thrice. Each value represents Mean±S.D, statistical analysis done by DMRT ($P \leq 0.5$).

Table 2. Inhibition Zone (mm) of Ethanolic leaf extract fractions against different Gram positive and Gram negative bacteria

Pathogen	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Streptomycin
B. cereus	15±0.23	26±0.23	5±0.23	2±0.65	40±0.53
B. subtilis	10±0.28	29±0.28	12±0.28	6±0.28	38±0.54
S. pyogenes	12±0.42	30±0.42	2±0.42	10±0.42	35±0.56
S. aureus	8±0.28	28±0.28	3±0.28	12±0.28	35±0.76
E. coli	5±0.23	24±0.23	8±0.23	6±0.23	40±0.53
P. mirabilis	10±0.28	15±0.28	10±0.28	3±0.28	38±0.54
K. pneumoniae	12±0.42	40±0.42	15±0.42	6±0.42	35±0.56
E. aerogenes	15±0.28	25±0.28	12±0.28	12±0.28	35±0.76

* Each test was repeated thrice. Each value represents Mean±S.D, statistical analysis done by DMRT ($P \leq 0.5$).

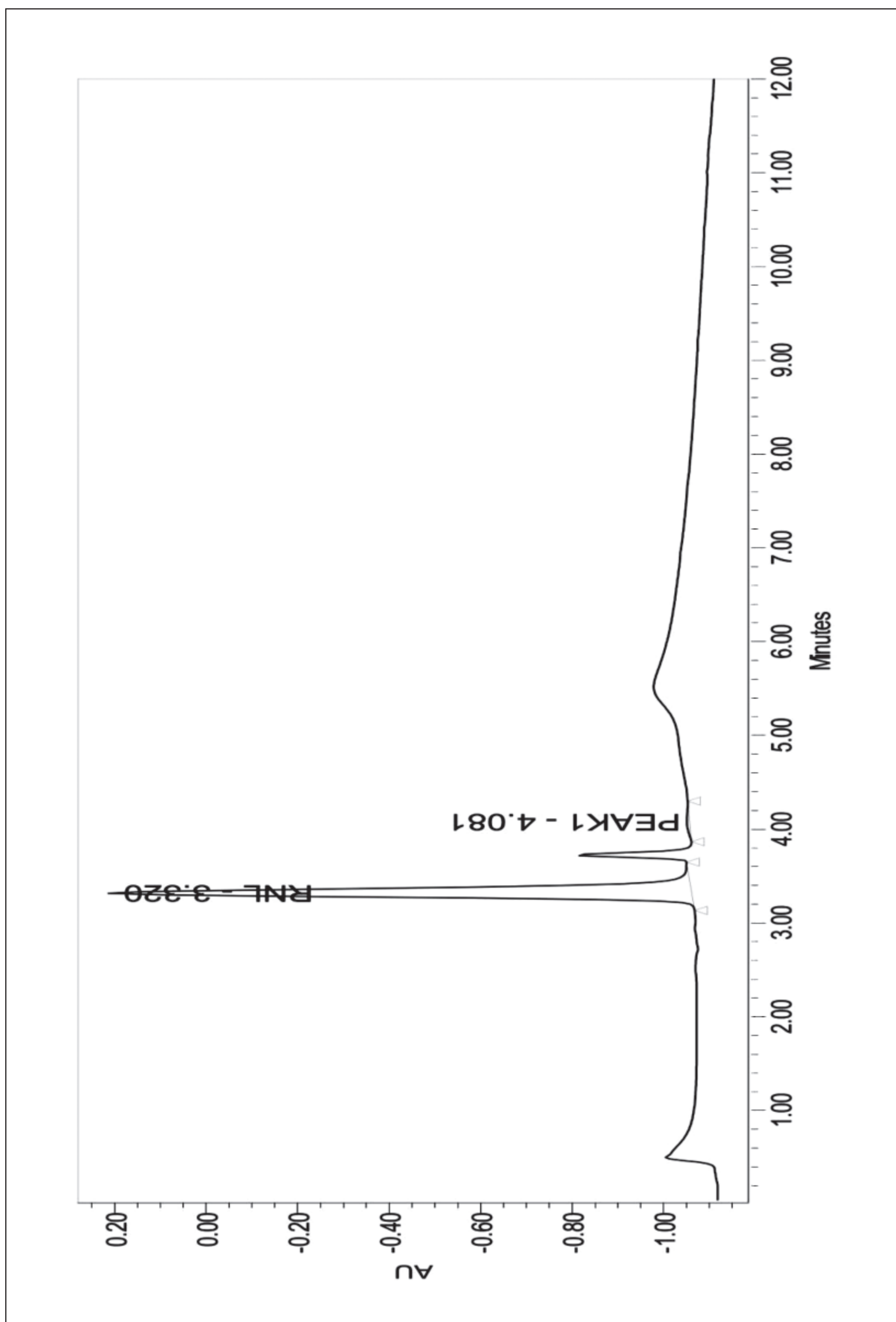


Figure 1. HPLC chromatogram of ethanolic leaf extract of *R. nasutus*.

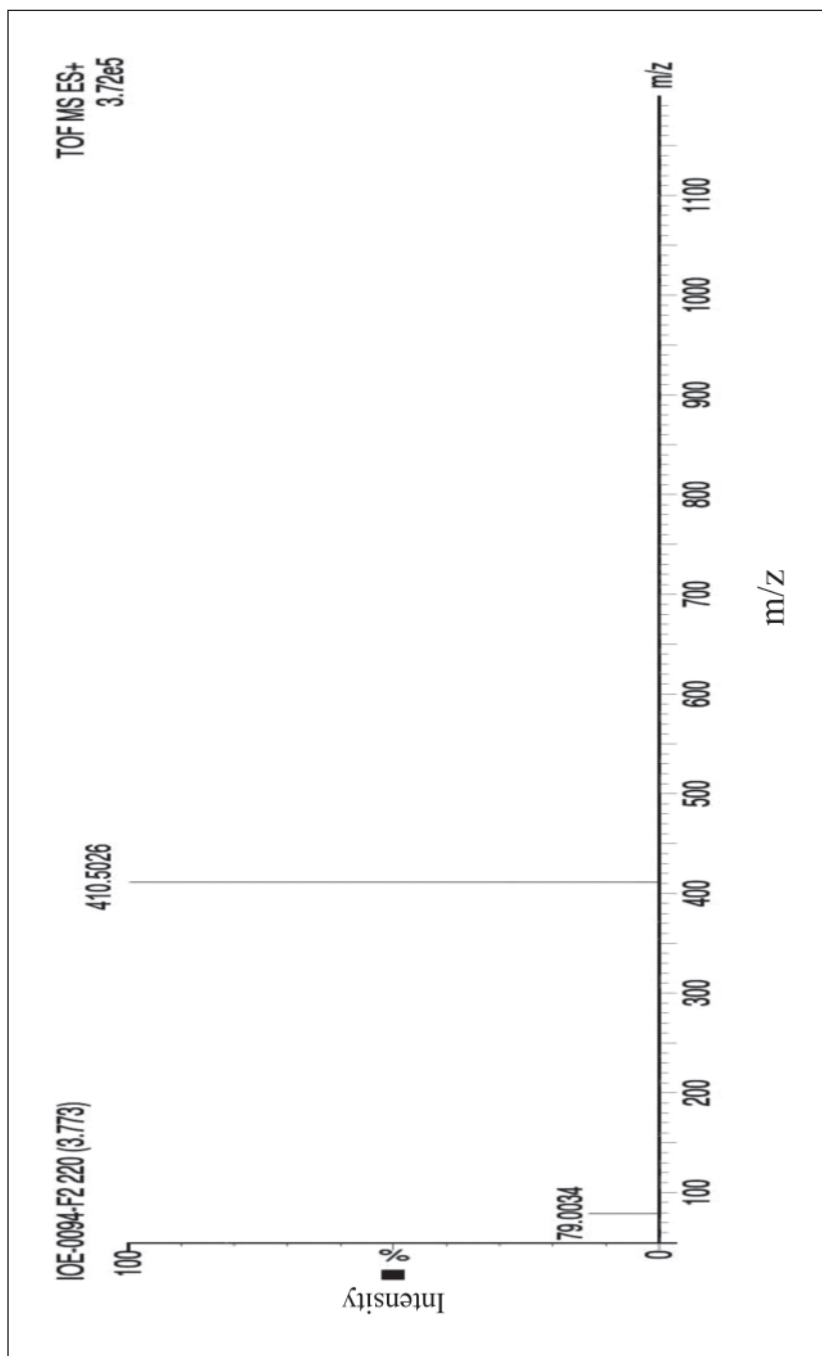


Figure 2. Mass spectrum of ethanolic leaf extract of *R. nasutus*.

cm^{-1}. The spectrum also reveals the presence of -CH bending at 1462.17 cm^{-1} and $-CH_3$ bending vibration at 1379.23cm^{-1} (Fig. 3).

The proton NMR (^1H-NMR) reveals the presence of 30 protons (six downfield signals, δ 5.82 – 8.71; and eight up field signals, δ 1.02 – 3.90). Signals at δ 8.98, δ 8.26, δ 7.99 and δ 7.71 suggest the naphthoquinone moiety. The signal at δ 6.67 reveals the presence of olefinic proton attached to the methylene signal (δ 2.10) which in turn is attached to other methylene signal (δ 2.05) (Fig. 4). In

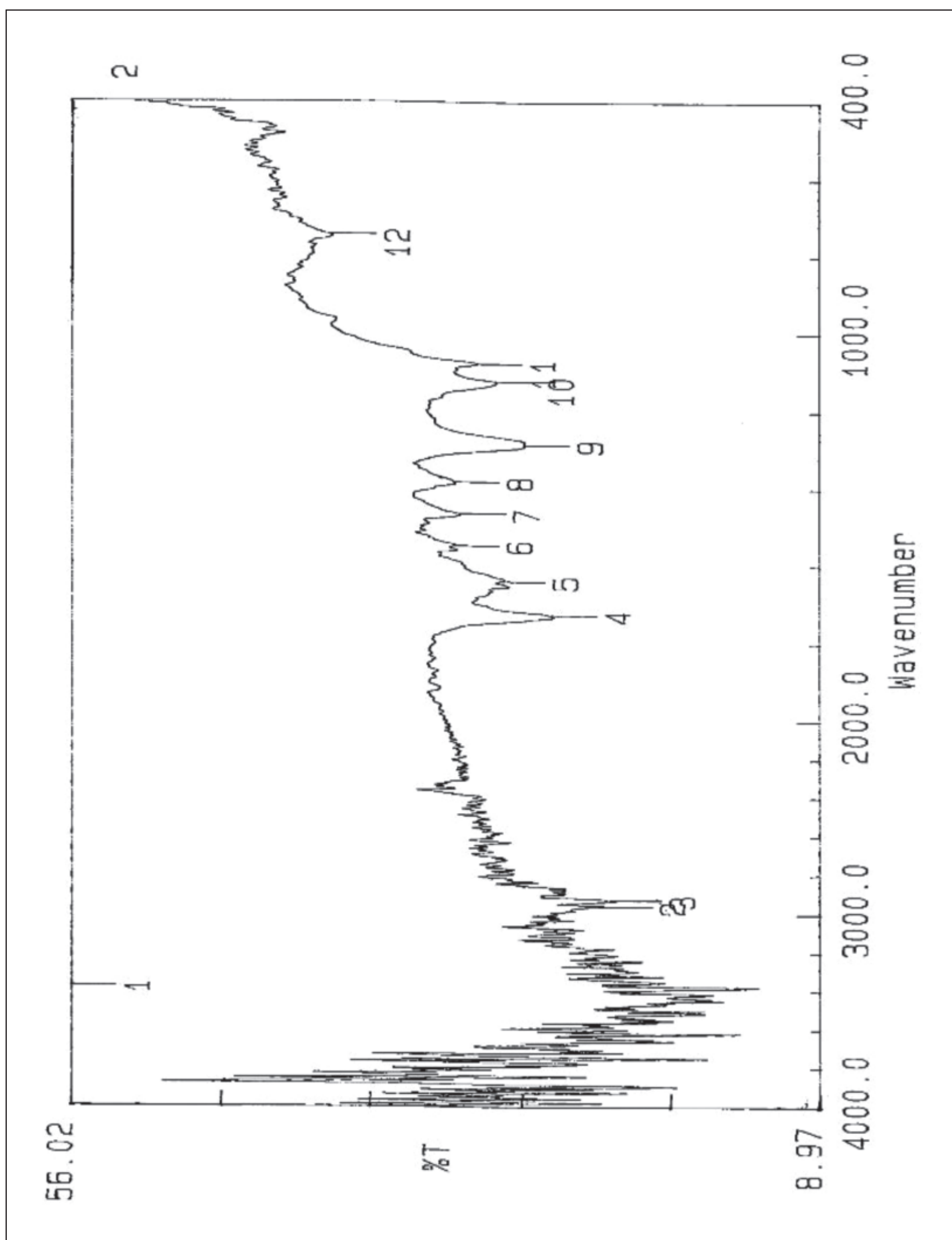


Figure 3. IR spectrum of ethanolic leaf extract of *R. nasutus*.

the ^{13}C-NMR spectrum, signals at δ 142.95, δ 134.65, δ 131.65, δ 133.65, δ 132.95, δ 128.45, δ 128.26, δ 127.65, δ 126.98 and δ 121.65 are consistent and represent aromatic ring carbons. Presence of aliphatic carbons is revealed by signals at δ 71.75, δ 55.62, δ 37.12, δ 35.34, δ 33. 25, δ 26.54, δ 26.64, δ 14.35 and δ 13.23. Signal at δ 55.62 reveals the presence of C-O linkage in the spectrum. Signals at δ 187.35 and δ 185.24 suggest C=O and the signal at δ 182.36 represents ester carbonyl group (Fig. 5). The spectral analysis

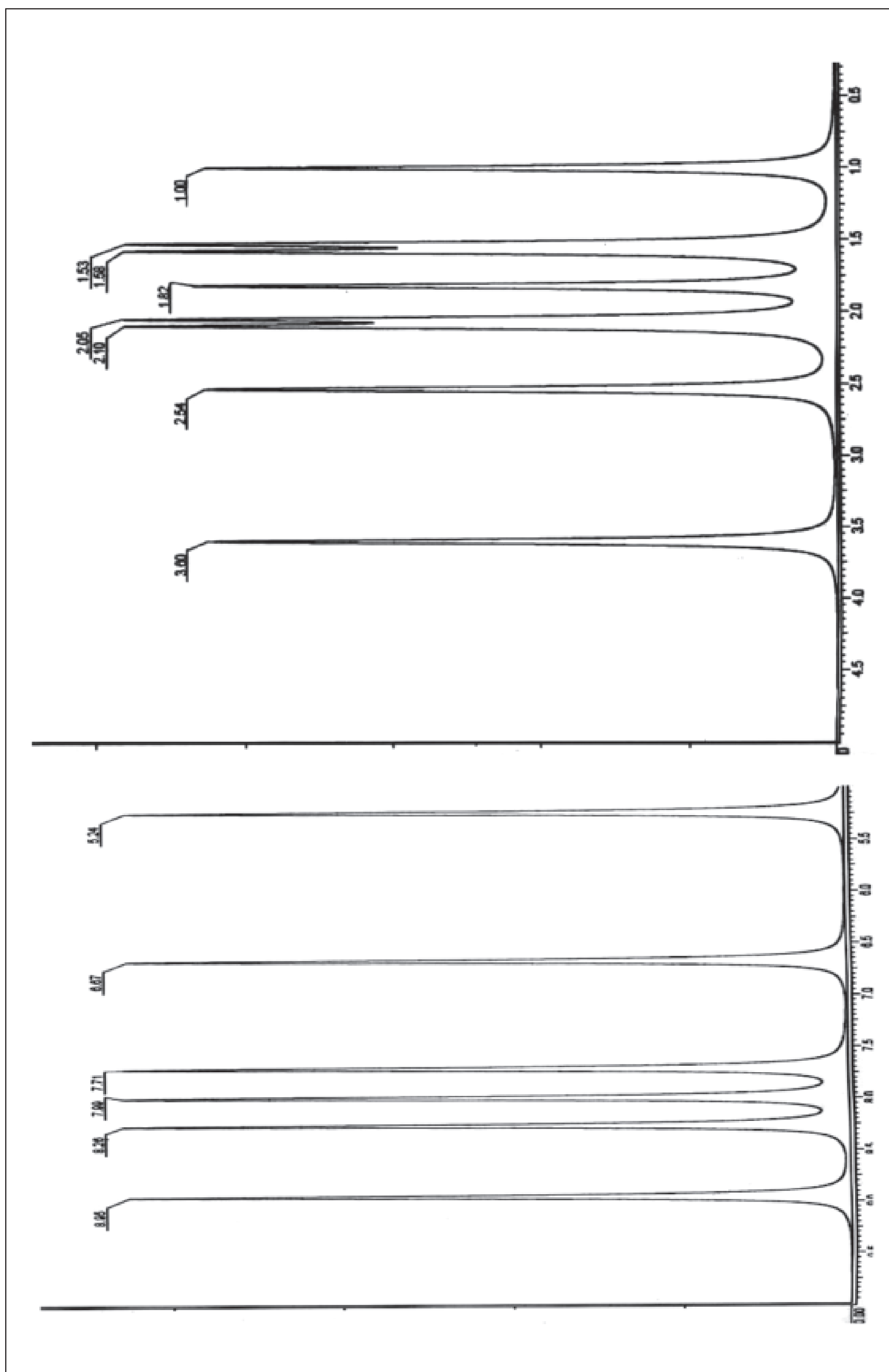


Figure 4. ^{1}H- NMR spectrum of ethanolic leaf extract of *R. nasutus.*

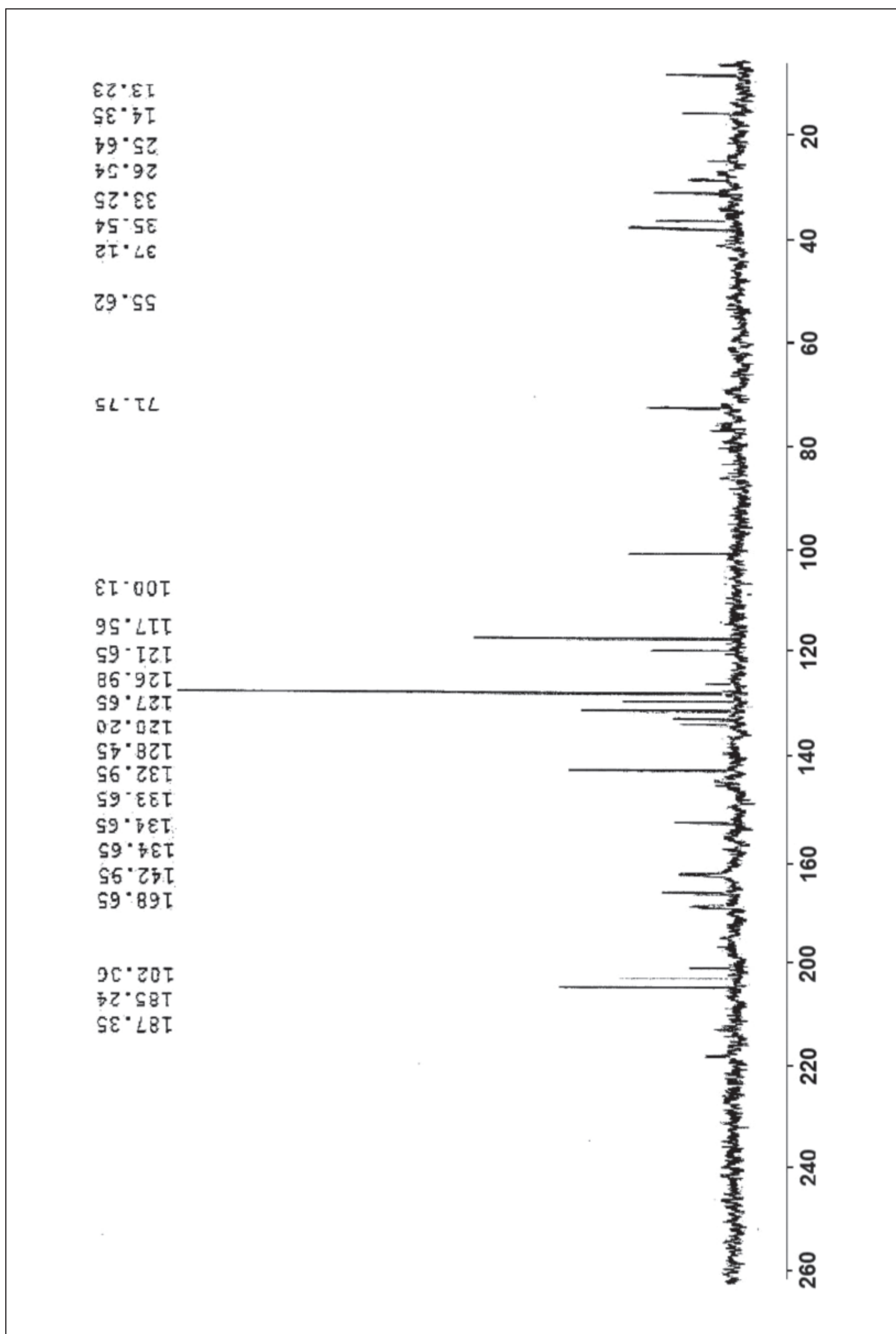
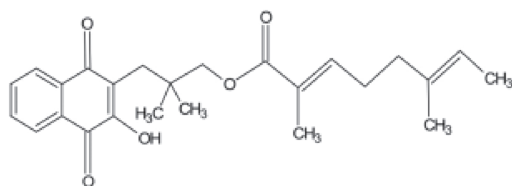


Figure 5. ^{13}C- NMR spectrum of ethanolic leaf extract of *R. nasutus*.

of the ethanolic fraction provides the data percentage as; C (73.15%), H (7.37%) and O (19.49%), which suggests the molecular formula as $C_{25}H_{30}O_5$.

The molecular ion peak of the sample found at 410 also corresponds to the molecular formula as $C_{25}H_{30}O_5$.

The purified compound has a spectral data alike to that of the rhinacanthin-C, a naphthoquinone, which has already been reported in earlier studies from the methanolic root extract of the same plant. The structure of the compound is as:



Structure of rhinacanthin-C

The IUPAC name of the rhinacanthin-C is 3-(3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-2,2-dimethylpropyl (2E,6E)-2,6-methylocta-2,6-dienoate.

Bioresources are considered as potentially diverse and rich resources of therapeutic agents. Phytoconstituents have a significant therapeutic property against diverse range of Gram positive and Gram negative bacteria (Lai and Roy, 2004). *R. nasutus* is extensively used in the ethnomedicine for the treatment of wide range of ailments including infectious diseases. The crude extract showed low antibacterial activity against the test bacteria compared to that of the individual pure fractions. This is attributed to the synergistic effect of the phytoconstituents. The same inference has been drawn by earlier authors (Thiyagarajan *et al.*, 2011). In the present study, the purified compound has shown the potent antibacterial activity against *K. pneumoniae.* The pathogen has been reported to have developed the resistance against the available antibiotic methicillin. As our study has confirmed the presence of potent bioactive compound and hence makes a scope for the development of broad spectrum antibiotic. Our findings justify the use of this plant in traditional medicine. Due to the easy redox cycling capacity, quinonoid compounds are known for their wide range of antimicrobial potential (Brandelli *et al.*, 2004). However, the potential antimicrobial property of quinonoid compounds has remained so far unexplored.

CONCLUSION

Phytoconstituents have played an important role in the lead drug discovery. In general, gram negative bacteria were more sensitive towards the crude extracts. The question remains to understand the mechanism of naphthoquinone antibacterial property and further *in vivo* studies need to carry out to strengthen the biological activity of the rhinacanthin-C.

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

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