

HPTLC-Based Quantitative Analysis of Scopoletin and In vitro Antioxidant and Antimicrobial Evaluation of *Hymenodictyon orixense* Roxb. Bark

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Abstract

Hymenodictyon orixense Roxb. is a medically valuable evergreen tree that has been traditionally used worldwide to treat conditions such as inflammation, infection, wounds, and chronic diseases. This study was conducted to evaluate the phytochemical analysis, in vitro antioxidant properties, antimicrobial activity, and HPTLC characterization of the ethanol extracts from the bark of *H. orixense* Roxb. by using scopoletin as a standard. The bark of *H. orixense* Roxb. was collected and identified as a medicinally valuable evergreen tree. This was dried under shade and Soxhlet-extracted using ethanol as the solvent. The preliminary phytochemical study of the ethanol bark extracts of *H. orixense* Roxb. identified the presence of alkaloids, flavonoids, phenols, tannins, saponins, steroids, glycosides, and fixed oils. The total content of the antioxidant compounds, like total phenols ($249.3 \pm 1.25 \mu\text{g GAE/mg}$), total flavonoid content ($81.29 \pm 0.56 \mu\text{g RE/mg}$), total tannin content ($47.85 \pm 0.44 \mu\text{g TAE/mg}$), and total alkaloid content (0.4% w/w) of the ethanol bark extracts of *H. orixense* Roxb. was determined. This study evaluated the antioxidant activity of ethanol extracts of *H. orixense* Roxb. using various in vitro assays, including the DPPH, ABTS, CUPRAC, superoxide scavenging, hydroxyl scavenging, and nitric oxide scavenging assays. The ethanol extracts of *H. orixense* Roxb. possessed key antioxidant activity in all the in vitro antioxidant assays. The studies revealed high DPPH scavenging activity and the least in ABTS scavenging activity, indicating higher electron donation and stronger scavenging of free radicals. The ethanol extracts of *H. orixense* Roxb. showed appreciable inhibition of *S. aureus* and *E. coli* with IC_{50} comparable to the standard chloramphenicol in disc diffusion methods.

1. Introduction

H. excelsum Roxb. Wall. ex-Mabb., also referred to as *H. excelsum*, is a deciduous tree that grows in the Rubiaceae family. It is known by local names such as Dendra Bandaru and Kala-bachnag in various areas of India and Thailand, where it is also referred to as U Lok and Som Kop, respectively. *H. excelsum* Roxb. grows in subtropical to tropical climates. It grows with a rounded top and a straight, cylindrical trunk that measures up to 10–12 meters in height. *H. excelsum* Roxb. has garnered attention in the fields of ethnomedicine and phytochemical studies due to its wide range of medicinal uses. The leaves, stems, roots, and flowers of *H. excelsum* have been used by traditional healers for the treatment of inflammation, wounds, infections, and chronic diseases such as arthritis and peptic ulcers. Well known in traditional medicinal systems of native societies for a considerable number of years, this species contains variable bioactive compounds, which may lead to beneficial uses in treating many ailments [1].

In traditional medicinal use, the bark extracts of this species serve as astringents, febrifuges, and antimyesthetics. Its wood is most effectively used in treating herpes. Its leaves have been used in the treatment of chickenpox, excessive sweating due to fever, toothaches, throat inflammation, mouth ulcers, prostate cancer [2], and tonsillitis [3]. Its antibacterial, anticoagulant, anti-inflammatory, and protective properties against UV radiation, known as sun-screening properties, have been well identified [4]. Its roots contain bioactive compounds like rubiadin, rubiadin methyl ether, lucidin, nordamnacanthal, damnacanthal, and 2-benzyl. The stem bark exudes hymexelsin, which is an apiglucoside compound

derived from scopoletin. The plant has been shown to contain other chemical constituents, including the iridoid glycoside loganin and four coumarins: scopoletin, scopolin, hymexelsin, and scopoletin 7-O- β -D-xylopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside. The plant extracts have been used in traditional medicine

The present investigation is to carry out a comprehensive phytochemical profiling of *H. orixense* Roxb. with quantitative estimation of Major secondary metabolites. In addition to evaluating the antioxidant efficacy against selected free radical scavenging assays and antimicrobial activity against selected Gram-positive and Gram-negative bacterial strains, thereby validating its ethnomedicine relevance. HPTLC will be used for quantification and fingerprints, ensuring standardization of the extract. The aim is to bridge traditional knowledge with modern analytical validation, providing a scientific basis for its therapeutic application and potential.

2. Materials and Methods

2.1 Plant collection and authentication

The bark of *H. orixense* Roxb. was collected from a local field in Chatgaon District, Gadchiroli, Maharashtra. The plant sample was scientifically identified and authenticated by Prof. Nitin Dongarwar from the Department of Botany at Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. A voucher specimen was created and stored in the Herbarium at Nagpur University, designated as sheet number 10071.

2.2 Chemicals and reagents

The reagents used in the study included 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid (GA), Cupric chloride (CuCl_2), ABTS, and trichloroacetic acid, all of which were purchased from SRL Chem Laboratories. Hydrogen peroxide (H_2O_2) was obtained from Neurochem Laboratories. Trichloroacetic acid (TCA), ferric chloride, and sodium nitroprusside were sourced from Fisher Scientific. Additionally, Ascorbic acid (AA), DMSO, EDTA, and trio-barbituric acid (TBA) were purchased from HiMedia.

2.3 Extraction of Plant Material

The plant bark was collected, decorticated, and cleaned of foreign particles. It was then air-dried in the shade to preserve its phytoconstituents and ground into a coarse powder. Once dried, the powder was stored in an amber glass container for further extraction. A hundred grams of this powdered bark was extracted with absolute ethanol using a Soxhlet apparatus. After extraction, the ethanolic solution was filtered, and the solvent was concentrated using a rotary evaporator.

3. Qualitative and Quantitative Assessment of Secondary Metabolites

The ethanolic bark extract of *H. orixense* Roxb. was analysed using standard qualitative phytochemical tests to identify its secondary metabolites. The results showed the presence of alkaloids (indicated by a creamy precipitate in Mayer's test), glycosides (red color in Borntrager test), flavonoids (red to pink in

Shinoda test), phenolic compounds (white precipitate in Gelatin test), steroids (red in Salkowski's test), tannins (reddish-yellow with nitric acid), fixed oils and fats (translucent stain in spot test), and saponins (formed foam in test) [5].

3.1 Total Phenolic Content

The total phenolic content (TPC) was measured using the Folin-Ciocalteu (FC) reagent, with gallic acid as the reference standard. A calibration curve was prepared with gallic acid (10–50 µg/mL). A stock solution of the extract (1 mg/mL) was made in Methanol and diluted for testing. Then, 1 mL of this solution was mixed with 0.5 mL of 2N FC reagent and 1.5 mL of 20% sodium carbonate. After 2 hours of dark incubation, absorbance was read at 765 nm using a Shimadzu UV-Visible spectrophotometer. TPC was expressed as mg of gallic acid equivalents per gram of extract, with all tests performed in triplicate [6],[7].

3.2 Total Flavonoid Content

Total flavonoids (TFC) were determined by the aluminium chloride colorimetric method. A 10 mg sample was dissolved in Methanol, then 2 mL of this solution was mixed with 20% methanolic aluminium chloride and acetic acid in a volumetric flask. After 40 minutes of incubation, absorbance was measured at 415 nm using a Shimadzu UV spectrophotometer. Rutin (0.5 mg/mL) served as the standard. TFC was expressed as mg rutin equivalents per gram of extract, with triplicate measurements [8].

3.3 Total Tannin Content

The total tannin content (TTC) was estimated using the FC reagent. A stock solution of ethanolic extract (1 mg/mL) was prepared and diluted to different concentrations (100, 200, 300, 400, 500 ppm). To each, 1 mL was mixed with 0.5 mL of FC reagent, followed by the addition of 1.5 mL of 20% sodium carbonate solution. Absorbance was measured at 775 nm after 15 minutes of incubation. Tannins were expressed in mg tannic acid equivalents per 100 g of extract (mg TAE/100g). All experiments were done in triplicate [9].

3.4 Total Alkaloid Content

Total alkaloids were determined gravimetrically. 5 g of powdered crude drug was extracted ultrasonically in three 50 mL portions with 0.1 M sulfuric acid. The filtered solution and washings were performed four times, discarding the supernatant each time, using 25 mL of dilute chloroform. Alkaloids were then extracted by making the solution basic with dilute ammonia. The basic solution was extracted with diethyl ether, filtered, dried, and weighed. Results were expressed as % w/w of the crude drug [6].

3.5 High Performance Thin Layer Chromatography

A stock solution of scopoletin at a concentration of 0.1 mg/mL was prepared using HPTLC-grade Methanol. An ethanolic extract of *H. orixense* Roxb. at a concentration of 5 mg/mL was also prepared by dissolving in MeOH and filtering through a whatman No. 01 filter. The above-mentioned solutions were

spotted uniformly on silica gel 60 F₂₅₄ thin-layer chromatography plates, which measured 200 × 100 mm. The scopoletin solution, at concentrations ranging from 2 to 16 µL, and the ethanolic extract, at concentrations ranging from 1 to 7 µL, were spotted using the CAMAG Linomat 5 applicator (vision CATS software) at a program rate of 150 nL/s, with every spot programmed to be 8.0 mm in length [10]. A mobile phase consisting of ethyl acetate, formic acid, and dH₂O. in the ratio of 6.7:0.5:2.6 V/V/V was used [11]. A 20 x 10 cm TLC plate was placed in a pre-saturated twin-tract glass chamber, along with the chosen mobile phase, for 20 minutes at room temperature. The development was done up to 70 mm solvent front using an automatic chamber. After that, the TLC plate was dried in air at room temperature for 10 minutes. The R_f value and spots of the separation were recorded. The dried plate was viewed under UV light at both 254 nm and 366 nm in a UV cabinet. All of the visible bands were recorded. Spectral scans were performed using a CAMAG TLC Scanner 4, equipped with deuterium and tungsten lamps that scanned wavelengths from 190 to 450 nm at a speed of 20 nm/s. Scanning was done track by track, and the R_f value of both substances was determined [10].

4. In Vitro Investigation of Antibacterial Potential Against *S. aureus* and *E. coli*

The antimicrobial activity of *H. orixense* Roxb. bark extract was tested using the Disk diffusion method against *S. aureus* (ATCC 23235) and *E. coli* (ATCC 25922). Mueller-Hinton Agar (MHA) was prepared and sterilized by autoclaving at 121°C and 15 psi for 10 minutes. Bacterial suspensions were adjusted to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). Wells with a 6 mm diameter were punched into the agar, and 10 µL of each sample—test samples, Chloramphenicol as a positive control, and Blank and distilled water as negative controls—were added. Plates were incubated at 35–37°C for 20–24 hours. Zones of inhibition were measured in triplicate, and average values were calculated [12]

5. In Vitro Antioxidant Assays

5.1 DPPH scavenging assay

5 µL of various concentrations of the test compound was added to 100 µL of a 0.1 mM DPPH solution in a 96-well plate. The reaction mixtures were prepared in triplicate for each concentration, with duplicate blanks created by adding 200 µL of DMSO/Methanol and 5 µL of the corresponding compound concentrations. The plate was incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a microplate reader (iMark, BioRad). Methanol served as the blank. A control reaction mixture containing 20 µL of deionized water was included. The radical scavenging activity (% inhibition) was calculated relative to the control, and IC₅₀ values were determined using GraphPad Prism 6 [13].

%Inhibition = $\frac{\text{Abs.}(\text{control}) - \text{Abs.}(\text{Sample})}{\text{Abs.}(\text{control})} \times 100$

5.2 ABTS scavenging assay

In a 96-well plate, 10 μL of test samples or ascorbic acid (standard) at different concentrations were added to 200 μL of ABTS reagent. After incubating in the dark at room temperature for 10 minutes, the absorbance was measured at 750 nm using a microplate reader (iMark, Bio-Rad). Control wells without treatment served as a baseline. Antioxidant activity was expressed relative to the negative control, and IC_{50} values were determined using GraphPad Prism 6 [14]

$$\% \text{Inhibition} = \frac{\text{Abs. (control)} - \text{Abs. (Sample)}}{\text{Abs. (control)}} \times 100$$

5.3 Hydroxy Free Radical Scavenging Assay

A reagent mixture was created by combining 10 μL of 0.5 M EDTA (HiMedia), 24.14 mg of Deoxyribose (SRL), 88 μL of FeCl_3 (10 mg/mL) and 28 μL of H_2O_2 (6%) (Neurochem Laboratories), then the volume was adjusted to 33 mL with water. Each well of a 96-well microplate received 10 μL of plant extract, 24 μL of phosphate buffer (50 mM, pH 7.4), and 10 μL of ascorbic acid. The plate was incubated at 37°C for 1 hour. Wells without treatment served as the negative control, with Gallic Acid (SRL) as the positive standard. After incubation, 50 μL of 10% TCA (Fisher Scientific) and 50 μL of 1% TBA (HiMedia) were added to each well. The formation of a pink chromogen indicated the degradation of deoxyribose, and the absorbance was read at 540 nm. IC_{50} was calculated using GraphPad Prism 6 software [15],[16].

5.4 Super Oxide Anion Radical Scavenging Assay

Serial dilutions of the *H. Orixense* Roxb. extract was prepared at concentrations from 1 to 1000 $\mu\text{g/mL}$. Riboflavin, used as the reference standard, was similarly diluted from 1 to 50 $\mu\text{g/mL}$. The reaction mixtures were incubated in a 96-well microplate under ambient illumination for 30 minutes. After preparing the serial dilutions of the *H. orixense* Roxb. ethanolic extract (1 to 1000 $\mu\text{g/mL}$), Riboflavin was diluted to 1–50 $\mu\text{g/mL}$ as a standard. The mixtures were then added to pre-treated samples, mixed thoroughly, and incubated under the same conditions. Untreated wells served as negative controls. Absorbance was measured at 560 nm with an ELISA microplate reader (iMark, BioRad, USA). The IC_{50} was calculated using GraphPad Prism version 6 software [17].

$$\% \text{Inhibition} = \frac{\text{Abs. (control)} - \text{Abs. (Sample)}}{\text{Abs. (control)}} \times 100$$

5.5 Nitric Oxide Scavenging Assay

The assay involved preparing a reaction mixture with 50 μL of 10 mM sodium nitroprusside (Fisher Scientific), 40 μL of distilled water, and 10 μL of gallic acid (SRL). Wells with the reaction mixture but without gallic acid acted as controls. The mixtures were pre-incubated under light at room temperature for 15 minutes to promote nitric oxide production. Then, 100 μL of Griess reagent was added to each well, including controls. The plate was incubated for an additional 5–10 minutes at room temperature to allow the chromophore to develop and stabilize. Absorbance was recorded at 540 nm and 660 nm using a microplate reader (iMark, BioRad, USA). The IC_{50} was determined using GraphPad Prism version 6 software [18].

$$\% \text{Inhibition} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100$$

5.6 CUPRAC Assay

In this assay, 10 µL of the sample at various concentrations was added to specific wells of a 96-well plate, followed by the introduction of 200 µL of the reagent mixture. Reactions were performed in triplicate, with duplicate blanks containing either Methanol and the sample or the Trolox standard, all of which were incubated in the dark for 30 minutes. Control wells remained untreated. After incubation, absorbance was measured at 490 nm using an iMark microplate reader (Bio-Rad, USA), and IC₅₀ values were calculated using GraphPad Prism 6 [19]

$$\% \text{Inhibition} = \frac{\text{Abs. (control)} - \text{Abs. (Sample)}}{\text{Abs. (control)}} \times 100$$

6. Results and Discussion

The phytochemical screening of the bark extract of *H. orixense* Roxb. showed the presence of various active compounds. The presence of alkaloids was revealed through positive Wager's and Mayer's reactions. The flavonoids gave positive reactions with the Shinoda and lead acetate tests. The presence of tannic acid in the sample was detected using various reagents, including ferric chloride, lead acetate, acetic acid, and dilute iodine. On the other hand, the glycosides (Keller-Killian test), steroids (Salkowski test), carbohydrates (Molisch's test), and proteins (Biuret test) resulted in negative reactions; therefore, these components are not present in the sample. The total phenolic content (TPC) in the crude extract was found to be 249.3 ± 1.25 µg GAE/mg, where GAE stands for gallic acid equivalents per milligram of extract. Total flavonoid content (TFC) came out to be 81.29 ± 0.56 µg RT/mg of extract in value, where RT stands for rutin equivalents per mg of extract. The tannin content (TTC) was found to be 47.85 ± 0.44 µg TA/mg of extract, where TA represents tannic acid equivalents per milligram. The total alkaloid content was found to be 0.4% w/w of the total extract yield.

Ethanollic extract of *H. orixense* Roxb. (5 mg/ml) was subjected to phytochemical screening, which showed abundant polyphenolic compounds. Thereafter, HPTLC was performed. Scopoletin (0.1 mg/mL in Methanol) was used as the standard. Chromatographic fingerprinting was carried out on the chromatographic matrix using a mobile phase mixture of ethyl acetate, formic acid, and distilled water (6.7:0.5:2.6 v/v/v). Densitometric scanning was performed at wavelengths of 254 nm and 366 nm. Scopoletin yielded characteristic chromatograms from densitometric scanning at wavelengths of 254 nm and 366 nm. The compound had an R_f value of 0.802, which was identical to that of the standard. Quantitative estimation revealed that the extract contained quantifiable amounts of scopoletin, 535.9 µg/5 mg.

6.2 Antimicrobial Activity

The agar disc diffusion assay was used to evaluate the antibacterial properties of the *H. orixense* Roxb. plant extract against *S. aureus* and *E. coli*. The discs prepared with the plant extract (A) created visible inhibition zones. The positive control, chloramphenicol (Ab), actively inhibited the growth. The distilled

water (D) and the discs labelled B had no inhibition zones, thus making the negative controls devoid of antimicrobial properties. The results from the antimicrobial study shown in Fig. 2 indicated an average inhibition zone of 21 mm for *E. coli*, similar to that of chloramphenicol, with an inhibition zone of 21.67 mm. For *S. aureus*, the average inhibition zone formed was 22.66 mm, which is close to the inhibition zone of the positive control, at 23.33 mm. The results of the blank and solvent controls ensured that the antimicrobial activity originated entirely from the bioactive agents in the *H. orixense* Roxb. extracts, as no activity was found in the blanks and solvents, indicating that the components of the extracts were responsible for their activities.

In Vitro Antioxidant Assay

The ethanolic extract of the *H. orixense* Roxb. exhibited diverse levels of antioxidant activity in various in vitro methods, as shown in Fig. 1. The importance depicted in the table focuses on the fact that ABTS and CUPRAC exhibited the highest level of antioxidant activity, with low IC₅₀ values of 3.656 and 5.490 µg/ml, respectively, indicating a higher free radical scavenging potential. While DPPH at 45.690 µg/ml and superoxide at 13.700 µg/ml exhibited good levels of efficiency, Hydroxyl at 143.890 µg/ml and Nitric oxide at 207.200 µg/ml showed significantly lower levels of antioxidant activity. It can also be perceived that the sensitivity levels of the various assays vary, and the most sensitive assay among them is the ABTS. This reflects the fact that the extract possesses a higher level of electron-donating capacity and free radical scavenging capacity.

Discussion

The current research study investigated the phytochemical diversity and biological potential of the ethanolic bark extract of *H. orixense* Roxb. The Phytochemical screening revealed the presence of both primary and secondary metabolites, with notable abundance of Phenolics, Flavonoids, tannins and Alkaloids. In contrast, carbohydrates, proteins, glycosides, and steroids were absent in the ethanolic extract. Quantitative analysis showed a significant content of total phenolics, flavonoids and tannin. Phenolic compounds are widely recognised for their antioxidant potential as they effectively scavenge free radicals and inhibit oxidative stress through an electron or hydrogen donation mechanism. Flavonoids also contribute to cellular protection by scavenging reactive oxygen species and reducing free radical formation. The HPTLC fingerprinting and analysis of scopoletin were valuable in providing the chemical constituents of the extract. The presence of scopoletin, a coumarin derivative, was established through successful analysis. The established HPTLC analysis method was efficient and accurate and can be applied for quality control analysis. The high concentration of scopoletin was directly responsible for the antioxidant and antimicrobial properties established in this research, since scopoletin has been shown to have significant free radical scavenging and antimicrobial properties. The ethanolic bark extract exhibited remarkable antimicrobial activity against *E. coli* and *S. aureus*, with inhibition comparable to the standard antibiotic chloramphenicol. The observed broad-spectrum efficacy of both Gram-positive and Gram-negative bacteria suggests the presence of bioactive constituents capable of disrupting bacterial cell walls or interfering with nucleic acid synthesis, causing the

antibacterial activity. The in vitro antioxidant assays demonstrated that the extract possesses a strong capacity to scavenge free radicals, with variation observed among different assays. The Marked activity in the ABTS and CUPRAC assay reflects a high electron-donating and reducing potential, whereas the moderate scavenging observed in the DPPH and superoxide assays further substantiates the antioxidant efficacy of the extract. The comparatively lower activity against hydroxyl and nitric oxide radicals may be attributed to differences in assay sensitivity and specific reactivity of the phytoconstituents toward these radical species. Overall, the findings of this study establish *H. orixense* Roxb. bark as a rich source of biologically active compounds with significant antioxidant and antimicrobial properties. The presence of Scopoletin, along with abundant polyphenolic constituents, provides a scientific basis for its traditional medical use and highlights its potential application in the development of natural therapeutic agents.

Conclusion

The bark extract of *H. orixense* Roxb. has been comprehensively validated as a rich source of bioactive secondary metabolites, notably phenolics, flavonoids, tannins and alkaloids, which collectively have potent antioxidant and antimicrobial properties. The strong free radical scavenging activity is shown in ABTS and CUPRAC assays. It also showed significant antimicrobial inhibitory effects against *S. aureus* and *E. coli* in agar disc diffusion assay. HPTLC fingerprinting confirmed and quantified Scopoletin, ensuring the standardisation and quality control. Collectively, these results validate the traditional use of *H. orixense* bark and identify it as a promising source for phytopharmaceutical development. Further work should involve bioassay-guided fractionation, in vivo efficacy and safety studies, and formulation optimisation.

Abbreviations

DPPH: 2,2-diphenyl-1-picrylhydrazyl

ABTS: 2, 2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid

IC 50: 50% Inhibitory concentration

TPC: Total polyphenol content

TFC: Total flavonoid content

GAE: Gallic acid equivalent

RE: Rutin equivalent

TA: Tannic acid equivalent

HPTLC: High Performance Thin Layer Chromatography

FCR: Folin-Ciocalteu Reagent

Rf: Retention factor

RT: Room temperature

Declarations

Conflict of Interest

No

Author Contribution

P.I. and S.P. conceptualised and designed the study. N.C., S.M., A.G., and S.P. carried out the experimental work and data collection. M.M. performed data analysis and interpretation. A.B. and P.I. drafted the original manuscript. S.P. and S.M. critically reviewed and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Figures

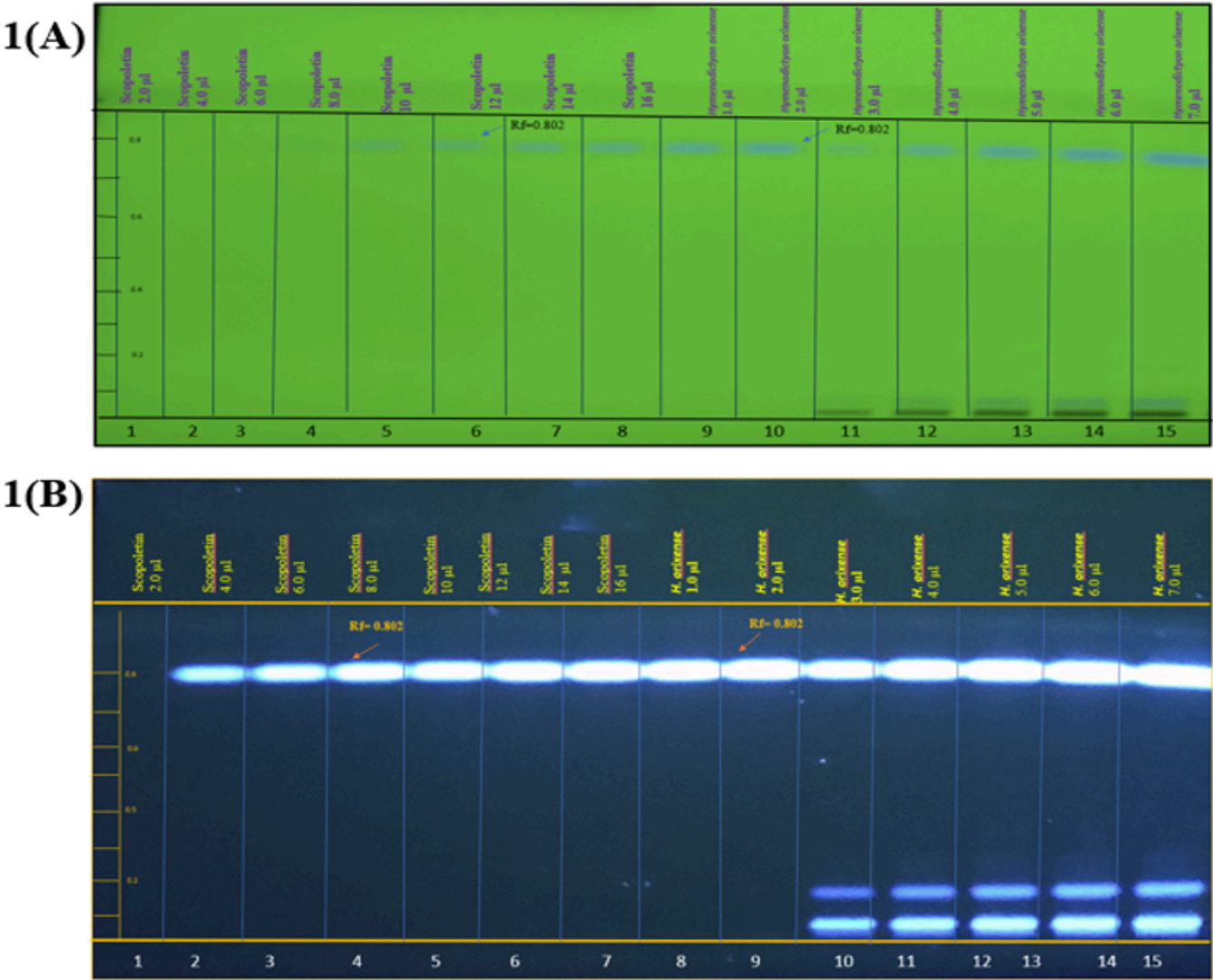


Figure 1

(A) HPTLC scanned plate at 254 nm and (B) HPTLC scanned plate at 366 nm

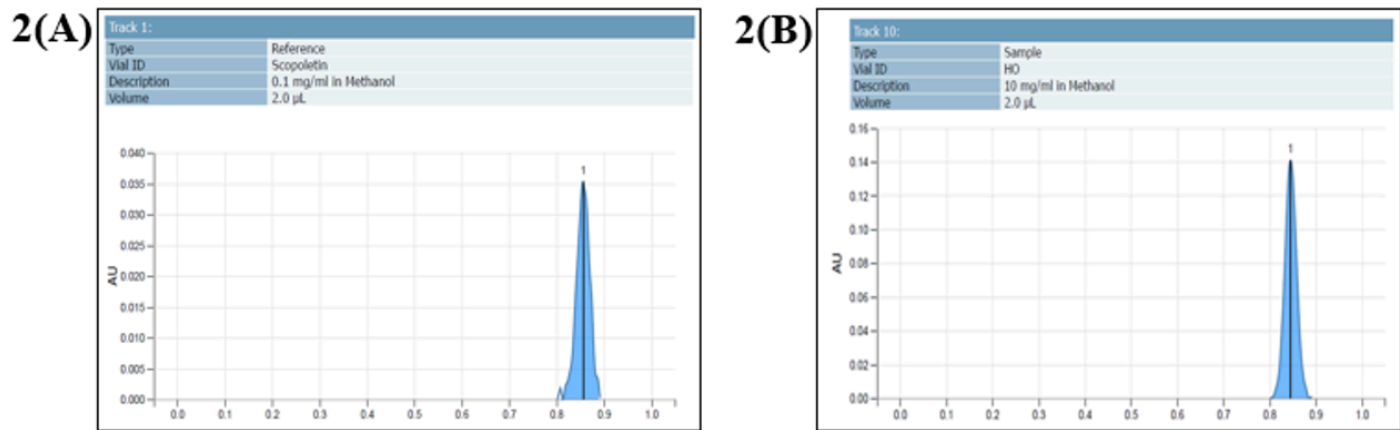


Figure 2

(A): HPTLC chromatograms of scopoletin and (B):): HPTLC chromatograms of H.orixense

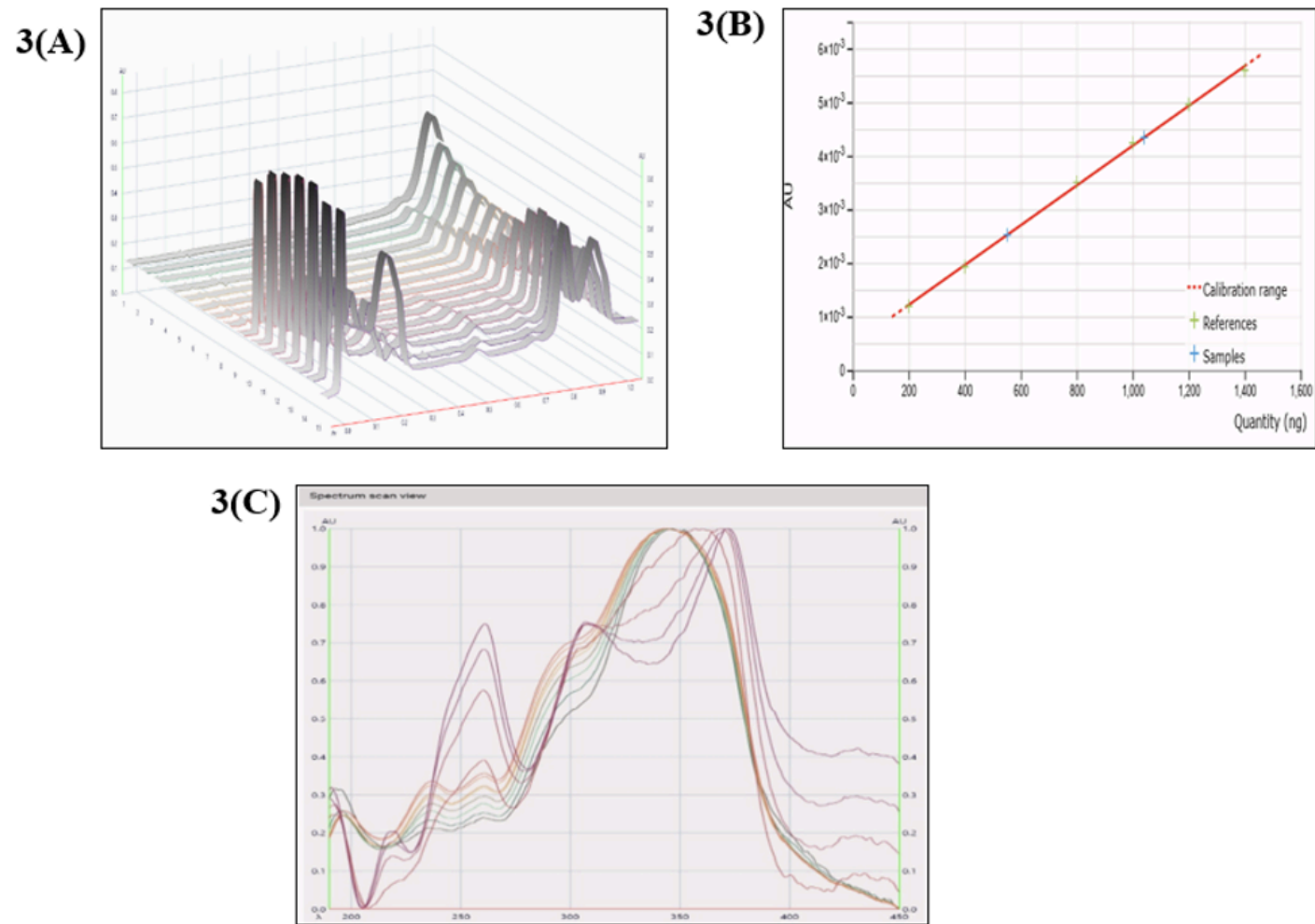


Figure 3

(A): 3D Densitometric Surface Plot, (B): Calibration Curve of Scopoletin and (C)UV-Visible Spectral Overlay

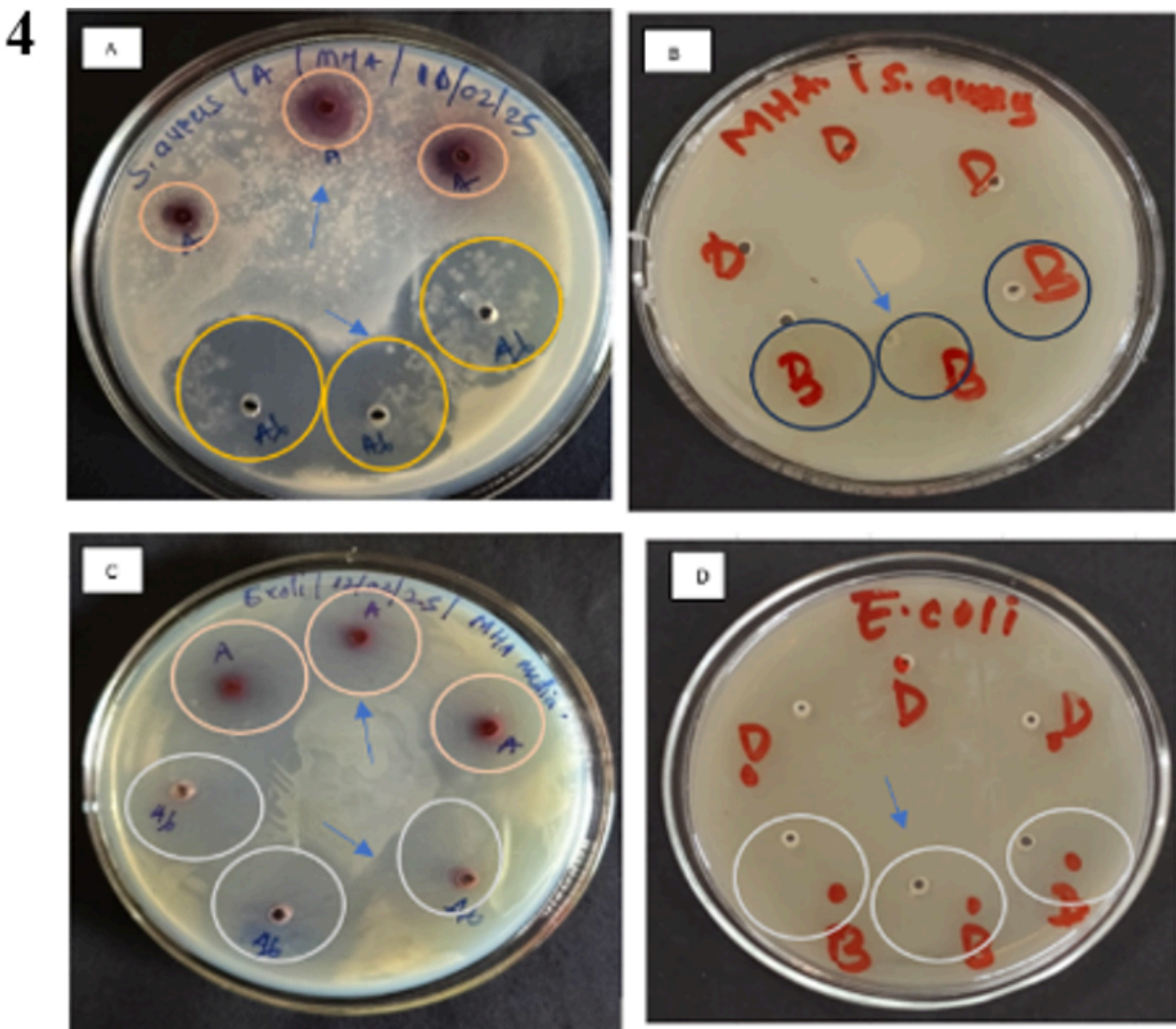


Figure 4

Zone of inhibition observed in disc diffusion assay

5

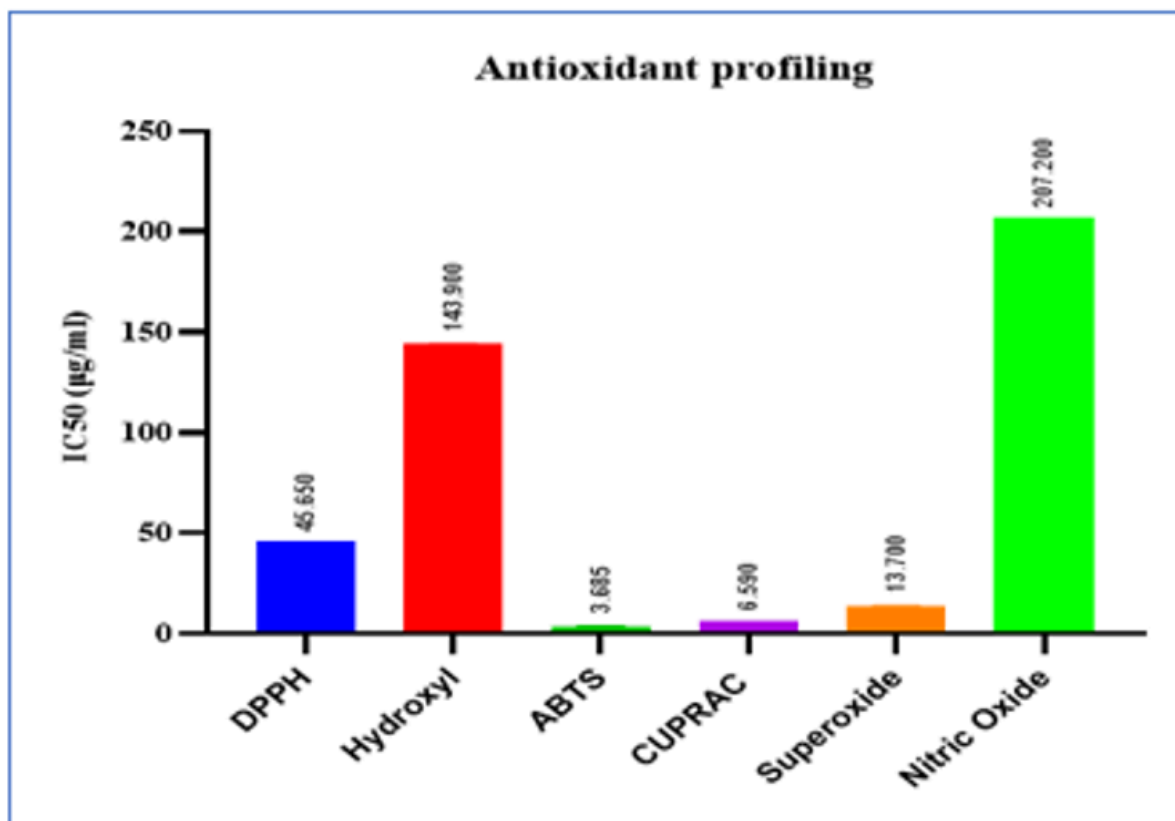


Figure 5

Comparative Antioxidant Activity of *H. orixense* Roxb. Bark Extract