ISOLATION AND CHARACTERIZATION OF CHEMICAL COMPOUNDS FROM THE STEM BARK FRACTIONS OF *CITRUS GRANDIS* AND EVALUATION OF THEIR ANTIMICROBIAL, CYTOTOXIC, AND ANTIOXIDANT ACTIVITIES

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ABSTRACT

Citrus grandis belongs to the Rutaceae family and is a naturally occurring citrus plant. The fractionated crude extracts of *C. grandis* have been studied for the investigation of its phytochemicals and allowed for the evaluation of biological activities, especially antimicrobial, cytotoxic, and antioxidant activities. At first, we assessed the antibacterial and antifungal properties using the disc diffusion method and conducted cytotoxicity screening using the brine shrimp lethality bioassay. Among the raw extracts, we isolated two compounds named CG-F3-B1 and CG-F3-B2 through column chromatographic separation of the chloroform extract. By comparing their proton NMR spectra with published spectra, we identified the chemical structures of these compounds as Zeylenol and Limonin, respectively. Regarding antibacterial testing, we observed that the methanol soluble fractions and petroleum ether fractions exhibited mild to moderate activity against bacteria. Additionally, the ethyl acetate soluble fraction showed moderate antibacterial activity. In addition, the crude extract displayed moderate antifungal activity against specific fungi. Furthermore, the cytotoxic assay revealed a relevant cytotoxic effect of a crude extract with an LC_{50} value of 37 µg/ml. The antioxidant activity was determined using the DPPH assay, and the methanol soluble extract exhibited modest antioxidant activity with an IC_{50} value of 89 µg/ml. Our findings demonstrated that the stem bark fractions and isolated compounds from *C. grandis* exhibited various promising biological activities, including antibacterial, antifungal, cytotoxic, and antioxidant properties.

KEYWORDS: *Citrus grandis,* Medicinal plant, Zeylenol, Limonin, Antimicrobial activities, Cytotoxic activities, Antioxidant activities.

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Introduction

Medicinal plants are those which have been utilized throughout human history for the treatment of different diseases. The Rutaceae plants contain a wide range of chemicals and unique pharmacologically active compounds which can be used for various biological and medicinal purposes (Chung S. K., 2000; Carvalho et al., 2022). Available research articles demonstrated that Rutaceae family plants contain a lot of bioactive phytochemicals which can perform antimicrobial, antioxidant, antitumor, and cytotoxic properties (Bocco et al., 1998; Palasuwan et al., 2005; Anmol et al., 2021). *Citrus grandis* (other name *Citrus maxima*) is a natural citrus plant that is used for the treatment of jaundice fever. Fruit juice of *C. grandis* is nutritious for human health and is taken to prevent diseases including jaundice, diabetes etc. Different types of electrolytes, including sodium (Na), potassium (K), phosphorous (P), zinc (Zn), manganese (Mn) and a lot of vitamins including vitamin C, riboflavin, niacin, thiamin, vitamin B_6 and B_{12} are also found in the fruit juice of *C. grandis* (Ghani, 1998; Shao et al., 2017; Ali et al., 2019). *C. grandis* plant contains flavonoids which can protect reactive oxygen species (ROS) suggesting its potential antioxidant activity (Heim et al., 2002; Franke et al., 2004).

Citrus fruit is rich with many poly nutrients which can be used for the treatment of diseases and for the health growth and promotion. Phytochemical ingredients were found in *C. grandis* that can replace natural preservatives. So these chemicals can be used in the fields of medicine, food industry and many other companies (Cowan, 1999; Abudayeh et al., 2019). Besides, numerous studies were undertaken to enhance the effectiveness of *C. grandis*. One study proposed that the effectiveness of

Pericarpium of *Citri Grandis* could be influenced by honey treatment (Hou et al., 2000). Moreover, the consumption of freshly squeezed red pummelo juice proved to be an outstanding source of antioxidant compounds, demonstrating remarkable efficiency in neutralizing various types of free radicals, including DPPH, superoxide anion, and hydrogen peroxide radicals (Tsai et al., 2007). To analyze the volatile constituents of cold-pressed peel essential oils, the redblush grapefruit (*Citrus paradisi*) and pummelo (*C. grandis* Osbeck) from the same region in Kenya were subjected to GC and GC−MS techniques, identifying a total of 67 and 52 compounds, respectively (Njoroge et al., 2005).

Moreover, a novel flavone called honyucitrin and a new coumarin named honyudisin were discovered in an acetone extract derived from the root bark of *C. grandis* Osbeck (Wu et al., 1988; Tian et al., 2019). Alongside these findings, nine known coumarins and 11 acridone alkaloids were also identified. The determination of their structures involved the utilization of spectral methods and certain chemical transformations. The antimicrobial properties of these compounds were evaluated as well (Wu et al., 1988). In addition, a comprehensive investigation was conducted on thirty-four varieties of citrus essential oils and their individual components to assess their efficacy in scavenging radicals. This assessment was carried out using the HPLC method with 1,1-diphenyl-2-picrylhydrazyl (DPPH) as the indicator (Choi et al., 2000; Lu et al., 2019).

Furthermore, the disk diffusion technique and determination of minimum inhibitory concentration (MIC) revealed that the extract derived from *C. grandis* displayed a broad range of antimicrobial properties against *E. coli*, *Staphylococcus aureus*, *B. subtilis*, *Saccharomyces cerevisiae*, and *R. oryzae* (Tao et al., 2010). Additionally, it was demonstrated that the fruit extract of *C. grandis* Osbeck triggers apoptosis in U937 cells (Lim et al., 2009). The overall phenolic content of the pomelo cultivars also exhibited a substantial correlation with both ferric reducing antioxidant power and Trolox equivalent antioxidant capacity. These findings imply that pomelo offers remarkable health advantages and holds potential for the development of functional foods (Mäkynen et al., 2013).

Compelling evidences also proved that *C. grandis* is a good source of natural antioxidants or phytochemical antioxidants which show antioxidant activities (Mokbel and Hashinaga, 2006; Kumar et al., 2019). Minor components of *C. grandis* ethyl acetate extract have synergistic activity on β-sitosterol and fatty acid (Jayaprakasha et al., 1997). Pummelo has furthermore demonstrated the presence of coumarins, furocoumarins, flavanones, flavones, and flavonols in both their unconjugated and glycosidic forms (Bocco et al., 1998; Zhang et al., 2011). Numerous investigations on the extract derived from pomelo have documented its advantageous antioxidant characteristics, specifically its ability to scavenge free radicals *in vitro* (Chang and Azrina, 2016; Kumar et al., 2019).

Based on the aforementioned previous studies, it is apparent that *C. grandis* exhibits promising bioactivity. However, a more comprehensive phytochemical characterization of the stem bark fractions of *C. grandis* is needed, particularly within the context of Bangladesh. Consequently, the primary objective of this study was to explore the phytochemical characteristics of the stem bark fractions of *C. grandis* and assess their antimicrobial, cytotoxic, and antioxidant activities.

Materials and Methods

Sample collection and preparation

The stem bark of *Citrus grandis* was obtained from Kazipur, located in the Sherajgonj district of Bangladesh. After that it was identified taxonomically by a botanist in University of Dhaka. After proper identification, the stem barks of *C. grandis* were cut into very small pieces which were 1.75 kg in weight. Then sun drying process was done which made it suitable for grinding purpose. After sun drying it was 700 gm in weight. Powdered formed sample was stored for further use at proper condition such as in dark and cold environment with air tight condition.

Isolation of chemical compounds from plant extracts

A total of 700 grams of sun-dried stem bark from *C. grandis* was pulverized and subjected to sequential extraction with petroleum ether, ethyl acetate, and methanol. The extraction procedure from the stem bark followed established protocols described in previous publications (Haque et al., 2022). To prepare a paste, the ethyl acetate extract was combined with silica gel, which was subsequently dried using a Buchi rotavapor. The resulting paste was subjected to flash column chromatography on silica gel (Kieselgel GF_{254}, Merck). The column was eluted with petroleum ether initially, followed by progressively increasing proportions of ethyl acetate in petroleum ether, and finally with methanol, resulting in the separation of several fractions. Specifically, a mixture of petroleum ether and ethyl acetate in a ratio of 98:02 was employed as the solvent system to obtain two compounds (CG-F3-B1 and CG-F3-B2) from the fraction number 3.

Structure determination using ^{1}H NMR spectroscopic data analysis

After screening and isolating compounds these were selected for ^{1}H NMR analysis. Then structure determination was done using proton NMR spectra analysis according to the established procedure (Haque et al., 2022). After screening all the fractions with TLC and PTLC fraction CG-F3-B1 and CG-F3-B2 were selected for Nuclear Magnetic Resonance (^{1}H NMR) analysis. For isolation selected sample was collected with silica gel by spatula and subjected into fresh vial tube and soaked with chloroform. Finally, two fractions were prepared for ^{1}H NMR.

Investigation of antimicrobial activities

The determination of antibacterial and antifungal activities of the crude extracts and purified compounds was carried out through the utilization of disc diffusion methods, following established procedures (Bauer et al., 1966) (Mahmud et al., 2021). Three primary fractions were subjected to antibacterial assays, namely the Petroleum Ether fraction (PEF), Ethyl Acetate Fraction (EAF), and Methanolic Fraction (MF). A total of thirteen bacterial strains and seven fungi were sourced from the Department of Microbiology and the Institute of Nutrition and Food Sciences at the University of Dhaka. Bacterial cultures were grown on nutrient agar media, while potato dextrose agar (PDA) media was employed for fungal cultures. Activity of crude extract against gram-positive bacteria were assessed using a standard streptomycin disc (10 µg/disc),

whereas activity against gram-negative bacteria were evaluated using a kanamycin disc (10 µg/disc) as positive controls. Negative controls consisted of blank discs absorbed with and dried $CHCl_3$. For antifungal activities, griseofulvin (25 µg/disc) served as the standard positive control. The antimicrobial activities were determined by measuring the zone of inhibition, expressed in millimeters.

Brine Shrimp lethality assay

The assessment of cytotoxic activities was conducted using the Brine shrimp lethality test, following established protocols outlined in prior studies (Meyer et al., 1982; Islam et al., 2015). First of all, nauplii is needed for Brine Shrimp lethality bioassay. For getting nauplii, Brine Shrimp eggs are hatched in sea water at proper condition for 24 hours. Then 10 numbers of Brine Shrimp are taken in a vial containing 10 ml of seawater. Sample solutions are made by adding calculated DMSO into test tube properly. Then samples of different concentrated are given into test tube with micropipette. Now test tubes are kept proper condition for 24 hours for survival. Then LC_{50} of test samples was determined by plotting of percentage of dead shrimp against logarithm of the sample concentration.

Assessment of antioxidant activity

Antioxidant assessment was done using DPPH assay as described (Kedare and Singh, 2011; Mandal et al., 2022; Shethi, 2022). DPPH is a stable radical in a solution. This is purple in color and absorbance frequency is 515 nm in methanol. In this assay a hydrogen atom is accepted from the scavenger molecules (antioxidant). As a result, reduction is occurred and DPPH is converted into $DPPH_2$. Purple color of DPPH is converted to yellow due to reduction. The intensity of color is measured by spectrophotometry shows antioxidant property. For determining antioxidant activity test BHT (tert-butyl-1-hyrdoxytoluene) was used as positive control.

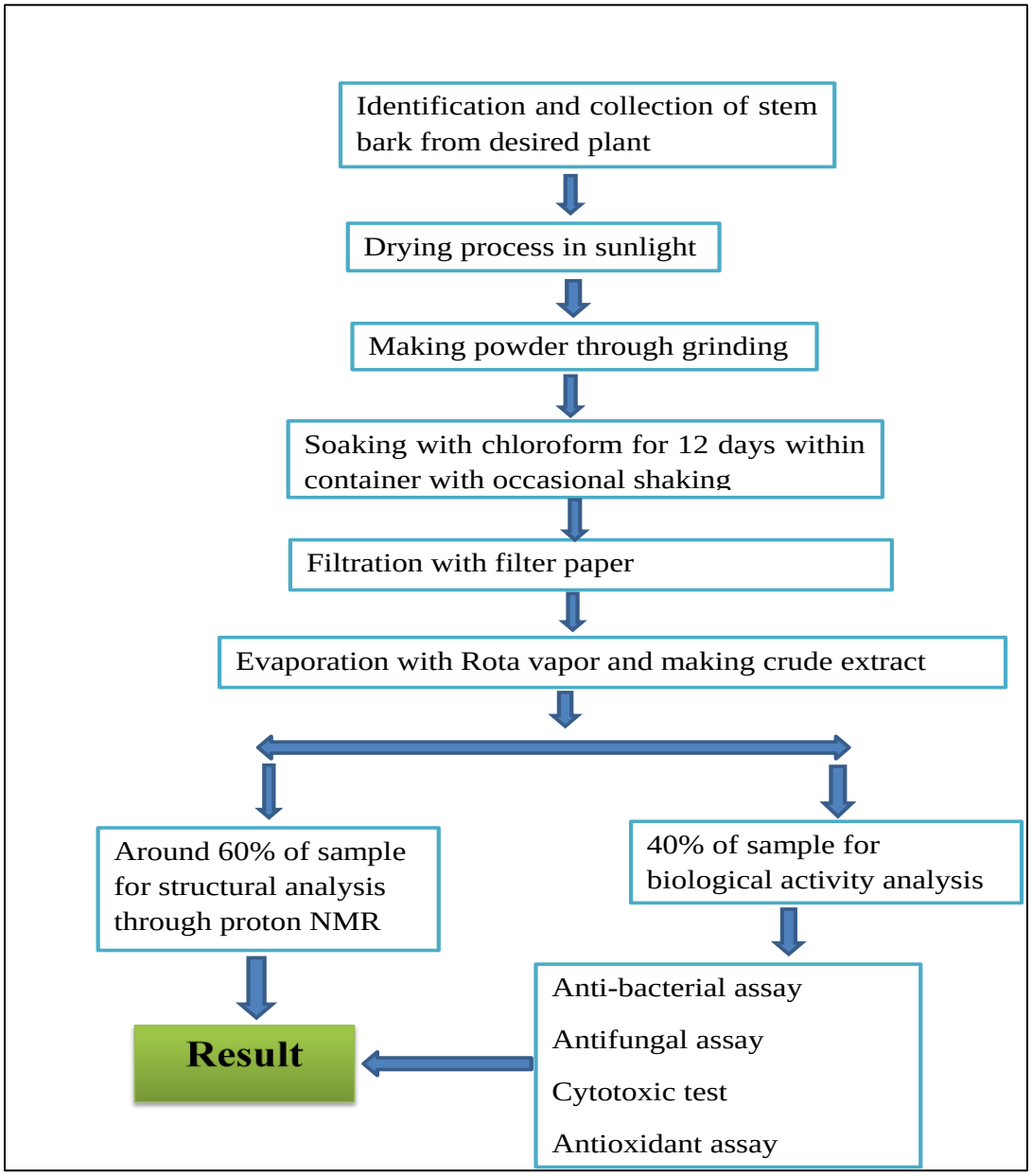

Figure 1. A flow chart showing step by step experimental procedures used in the study

Results

Isolation of chemical compounds

From the comparison of ^{1}H NMR data with published data it was demonstrated that, observed data resembled with Zeylenol (Table 1, Figure 2A). For the second compound, as these proton NMR spectra resembled with the published data of Limonin, therefore this compound was Limonin (Table 2, Figure 2B).

Table 1. Resembling proton NMR spectra of CG-F3-B1 with proton NMR spectra of Zeylenol

Position of proton	δH values	Published values
H-2	4.23 (1H,d, J 6.0 Hz)	4.25 (1H,d, J 6.2 Hz)
H-6	4.31 (1H,dd, J 3.5, 1.0 Hz)	4.32 (1H,dd, J 3.7, 1.0 Hz)
H-7a	4.60 (1H, d, J 11.5 Hz)	4.59 (1H, d, J 11.9 Hz)
H-3	5.75 (1H,ddd J1.6.5, 2.5, 6.0 Hz)	5.72 (1H,ddd J1.7, 2.6, 6.2 Hz)
H-4	5.84 (1H,ddd, J 10.02, 2.4, 1.01 Hz)	5.85 (1H,ddd, J 10.1, 2.6, 1.0 Hz)
H-5	6.01 (1H,ddd, J 10.01, 4.3, 1.8 Hz)	6.04 (1H,ddd, J 10.2, 4.1, 1.7 Hz)
H-3', H-3"	7.40 (4H, m)	7.42 (4H, m)
H-4', H-4"	7.55 (2H, m)	7.56 (2H, m)
H-2"/6"	8.01 (2H,d, J 7.6 Hz)	8.03 (2H,d, J 7.4 Hz)

Table 2. Resembling proton NMR spectra of CG-F3-B2 with proton NMR spectra of Limonin

Position of proton	δH values	Published values
18	1.16 (s)	1.17 (s)
25	1.24 (s)	1.25 (s)
12	1.55 (m)	1.46 (m)
11	1.69 (m)	1.78 (m)
11	---	1.87 (m)
2	2.26 (dd)	2.30 (dd)
5	2.40 (dd)	2.44 (dd)
6	---	2.73 (dd)
15	4.09 (s)	4.05 (s)
19	4.48 (d)	4.50 (d)
19	4.79 (d)	4.82 (d)
17	5.45 (s)	5.48 (s)
22	6.30 (d)	6.36 (d)
23	7.4a (d)	7.45 (d)

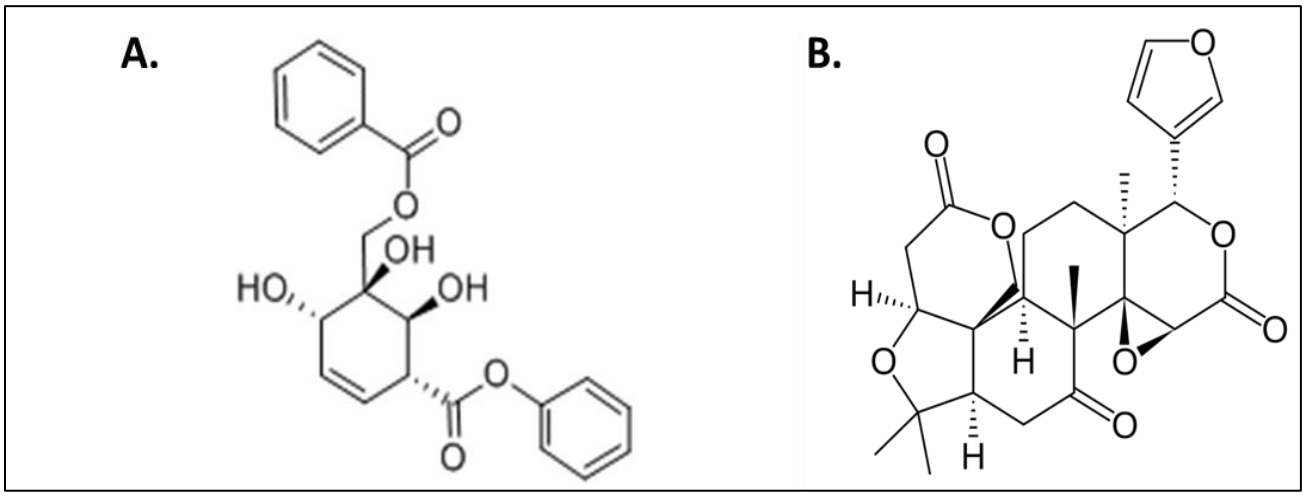

Figure 2. Isolation of two compounds named Zeylenol (A) and Limonin (B)

Antimicrobial activity of crude extracts

To evaluate antibacterial activity different fractions and crude extract have been tested against several bacteria by disc diffusion method which indicates resistance and sensitivity of bacteria to different extracts of plant stem bark. Upon careful examination, it was anticipated that the Petroleum Ether fraction (PEF) exhibited a moderate level of antimicrobial activity against both gram-positive and gram-negative bacteria. On the other hand, EAF and MEF showed lower antimicrobial activity compared to PEF. Range of diameter of zone of inhibition of PEF was 7-13 mm. On the other hand, range of diameter of zone of inhibition of standard disc was 10-17 mm. *Bacillus subtilis, Bacillus cereus, Enterobacter cloacae, and Escherichia coli* showed maximum sensitivity to the extracts (Table 3 and Table 4). However, *Lactococcus lactis, Salmonella typhi* showed minimum sensitivity.

Figure 3. Agar plates with sample and control

Table 3. Determination of antimicrobial activity against gram positive bacteria

Name of the used gram positive bacteria	Diameter of zone of inhibition in (mm)			
	PEF	EAF	MF	Streptomycin (30 µg/disc)
Bacillus cereus	13	-	-	15
Bacillus subtilis	10	7	8	12
Staphylococcus epidermis	7	-	-	10
Staphylococcus aureus	9	9	9	13
Bacillus polymyx	8	-	-	-
Lactococcus lactis	-	-	7	12
Clostridium botulinum	10	10	-	17

Table 4. Data for the determination of antimicrobial activity against gram negative bacteria

Name of the used gram negative bacteria	Diameter of zone of inhibition (mm)			
	PEF	EAF	MF	Kanamycin (10 µg/disc)
Escherichia coli	10	10	-	16
Enterobacter cloacae	12	-	7	17
Vibrio cholera	9	8	-	13
Salmonella typhi	8	-	-	-
Shigella dysenteriae	-	12	-	14
Pseudomonas sp.	9	10	12	12

Antifungal activity of crude extracts

Antifungal activity was also determined by Disk Diffusion Method although there are some differences in composition and process. In antifungal activity test, Potato and glucose were utilized but in antibacterial test these compounds were not utilized. Besides in antifungal activity test 48 hours' incubation at 37ºC was needed. After observation it has been demonstrated that crude extract showed prominent activity against *Candida albicans*, however, fraction 3 showed higher activity against *Rhizopus oryzae* (Table 5).

Table 5. Data of zone of inhibition of crude extract and F-3 fraction against Fungi

Name of fungal strains	Diameter of zone of inhibition (mm)		
	Crude extract	F-3 fraction	Griseofulvin (25 µg/disc)
Saccharromyces cerevisiae	8	6	12
Candida arrizae	-	7	10
Candela albicans	8	-	8
Rhizopus oryzae	7	9	11
Candida krusii.	-	-	10
Aspergillus niger	8	-	-
Candida albicans	10	8	11

Cytotoxic activity of crude extracts

For the measurement of the cytotoxicity of natural compounds Brine shrimp lethality bioassay was performed. During the brine shrimp lethality bioassay, it was observed that the crude ethyl acetate extract demonstrated significant toxicity towards the brine shrimp, as indicated in Table 6. Comparatively, the crude extract exhibited greater potency when compared to the selected fractions. Notably, the mortality rate of the brine shrimp increased correspondingly with the concentration of each sample. From the comparison LC_{50} graph after calculation, LC_{50} of control was around 19 µg/ml and sample was 37 µg/ml (Figure 4). Therefore, it has been demonstrated that crude sample has moderate cytotoxic effect on Brine Shrimp.

Table 6. Data for the determination of Log of concentration with mortality rate of Brine Shrimp

Test tube no.	Concentration (µg/mL)	Log of concentration	Percent (%) of mortality	
			Control	Crude extract
1	400	2.602	100	70
2	200	2.477	100	70
3	100	2.176	80	60
4	50	1.875	70	50
5	25	1.574	60	50
6	12.50	1.273	50	30
7	6.25	0.971	40	10
8	3.125	0.670	20	10
9	1.56	0.370	20	0
10	0.000	-----	0.0	0.0

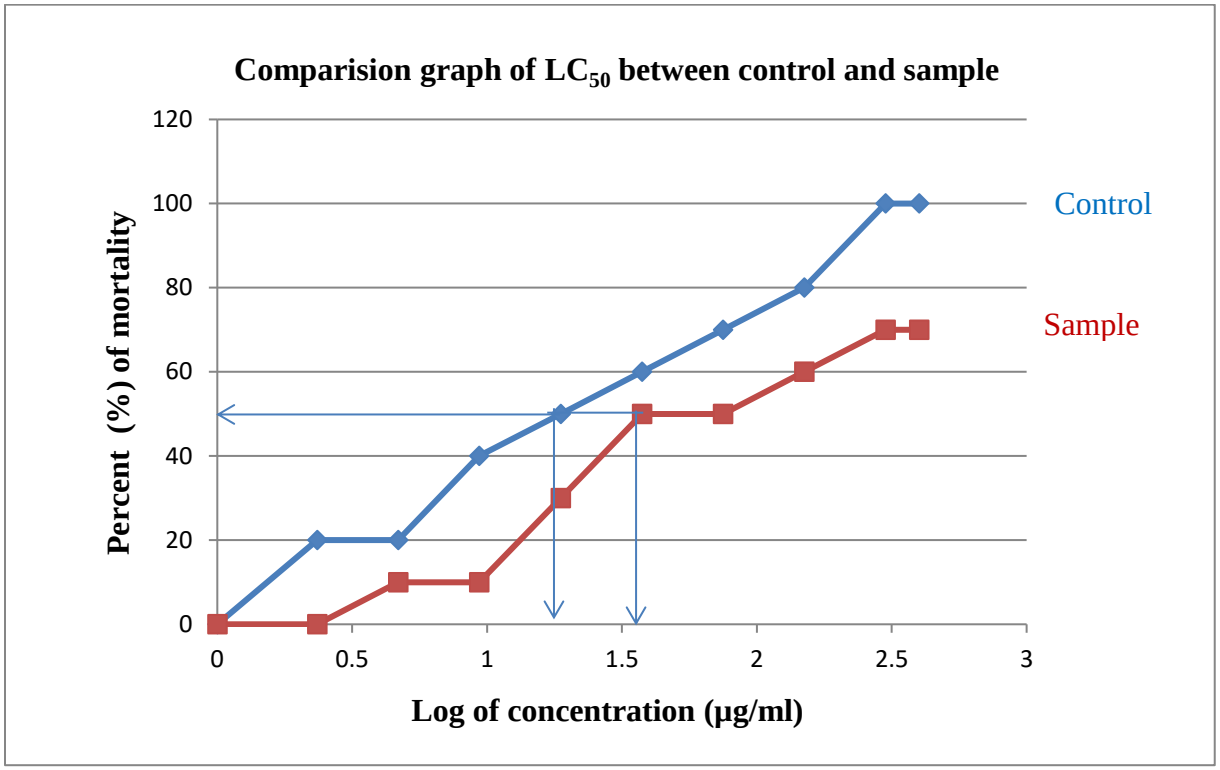

Figure 4. Graphical representation of the logarithm of concentration against the percentage of mortality

Determination of antioxidant activity

There are many types of antioxidant assay which are utilized for determining antioxidant properties of plant extract. Among these DPPH assay was adopted for the determination of the antioxidant activity of *C. grandis* crude extract. 2,2-diphenyl-1-picrylhydrazyl known as DPPH is a stable free radical. Physically it is dark colored crystalline powder. It is utilized as a main kit for antioxidant assay to assess the antioxidant activity of desired plant extract. Based on our investigation, it was noted that the crude extracts displayed a concentration-dependent ability to scavenge DPPH free radicals (Table 7, Figure 5). Notably, the ethyl acetate extracts exhibited the most pronounced activity in terms of free radical scavenging. From the comparison IC_{50} graph after calculation, IC_{50} of control was around 16 µg/ml and sample was 89 µg/ml (Figure 5).

Table 7. Data for the determination of percent of inhibition of crude sample and control

Concentration (µg/ml)	Absorbance of crude extract (nm)	Absorbance of control (nm)	Absorbance of blank (nm)	Percent (%) of inhibition of control	Percent (%) of inhibition of crude extract
500	0.017	0.092	0.346	95.08	73.41
250	0.060	0.136		82.65	60.70
125	0.089	0.149		74.27	56.93
62.5	0.100	0.192		70.80	44.50
31.25	0.139	0.220		59.82	36.416
15.625	0.169	0.250		51.15	27.74
7.813	0.198	0.312		42.77	9.82
3.906	0.215	0..333		37.86	3.76
1.953	0.232	0.341		32.94	1.45
0.977	0.278	0.00		19.65	0.00

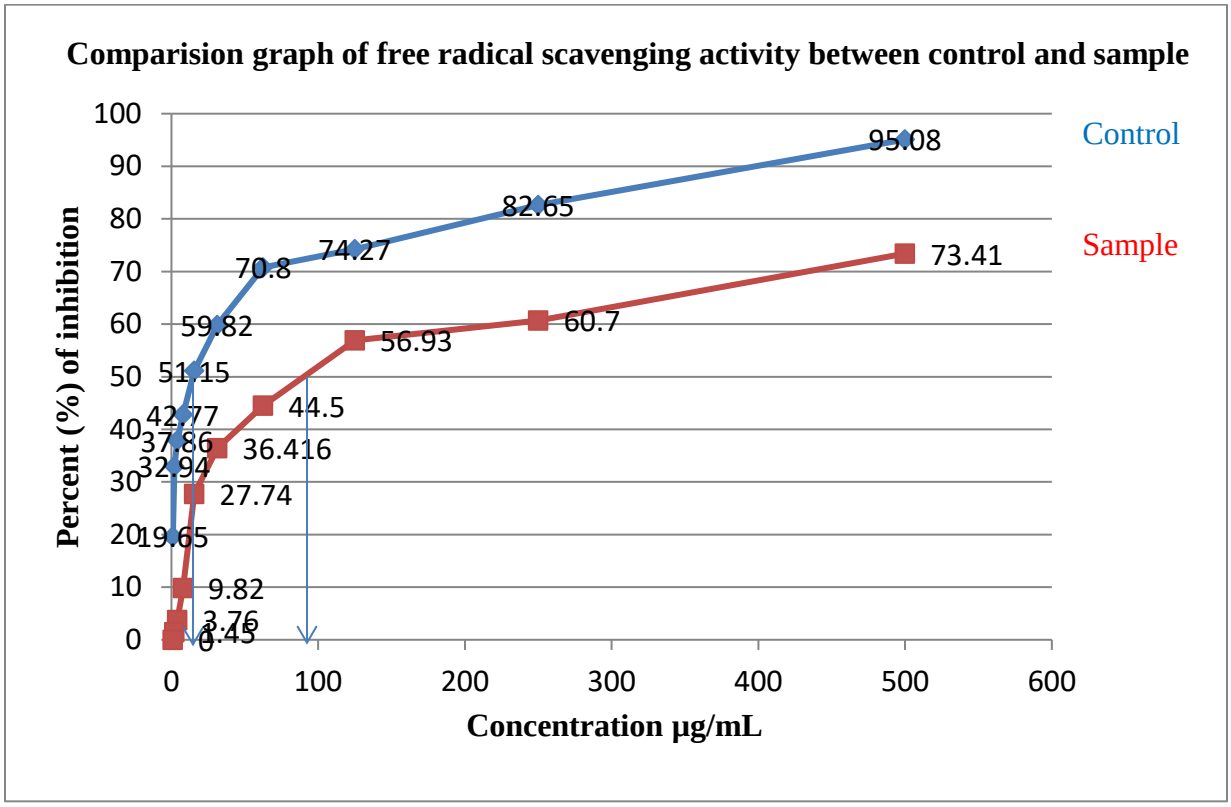

Figure 5. Graphical representation of the concentration against the percent scavenging activity

Discussion

Stem bark of *C. grandis* (Batabi lebu, jambura) plant was selected for phytochemical investigation and evaluation of antimicrobial, cytotoxic, and antioxidant activities. For assessment of biological activities including antimicrobial, cytotoxic and antioxidant activities of crude extract, PE extract, EA extract, ME extract, and different fractions found from fractionation were assayed. After fractionation, 28 fractions were found for further screening through TLC and PTLC. Several spots were found in F3 and F4 through TLC screening, but F4 spots were not clear like F3. As a result, F3 was allowed for final analysis through proton NMR. In F3 fraction, two spots were found clearly in middle and lower middle respectively. Finally, two spots were isolated and allowed for proton NMR analysis. After getting proton NMR spectra these spectra were analyzed with published NMR data. As a result, two compounds were identified which are Zeylenol and Limonin from CG-F3-B1 and CG-F3-B2 respectively.

The assessment of *in vitro* antibacterial activity for gradient extracts and isolated compounds was conducted utilizing the agar disc diffusion technique on a total of thirteen (13) distinct bacterial strains. Herein, different extract including PE extract, EA extract and ME extracts as well as F3 fractions were used. Zone of inhibition was measured and compared with control disk containing streptomycin and kanamycin antibiotics. PEF showed moderate antimicrobial activity against both gram positive and gram negative bacteria. On the other hand, EAF and MF showed lower antibacterial action compared to PEF. In case of gram positive bacteria, the highest zone of inhibition was around 13 mm against *Bacillus cereus* in PEF and the lowest zone of inhibition was around 7 mm against *Staphylococcus epidermis*. On the other hand, in case of gram negative bacteria the highest zone of inhibition was 12 mm against *Enterobacter cloacae* and the lowest was 8 mm against *Salmonella typhi*. Other fractions showed mild antibacterial activities against gram negative bacteria. Furthermore, crude extract of *C. grandis* stem bark was also assessed for antifungal activities. Crude extract showed prominent activity against *Candida albicans* on the other hand fraction 3 showed higher activity against *Rhizopus oryzae*. Therefore, the stem bark of *C. grandis* has mild to moderate antifungal activities against specific fungi.

In summary, after comparison of different extracts with control it can be revealed that stem bark of *C. grandis* has reasonable antibacterial function against both gram positive and gram negative bacteria. Moreover, purified extracts and fraction-3 obtained from stem bark of *C. grandis* has potential to be utilized as antifungal agent.

Stem bark of *C. grandis* was further taken for cytotoxic assay. For that purpose, Brine Shrimp lethality assay was performed to assess cytotoxicity of *C. grandis* stem bark. Crude sample revealed moderate cytotoxicity against Brine Shrimp. Vincristine sulfate was used here as positive control. From the experiment, LC_{50} value of control was 19 µg/mL and LC_{50} value of sample was 37 µg/ml which demonstrated the relevant cytotoxicity of the crude extract. As sample revealed moderate cytotoxicity so it can be demonstrated that *C. grandis* stem bark has moderate cytotoxic effect against living organisms. As a result, cytotoxic agent can be formulated from *C. grandis* stem bark.

Finally, crude sample from *C. grandis* was assessed for antioxidant activity. For performing the assay DPPH assay was selected to assess test crude samples. Here BHT was used as control. Crude extract showed considerable antioxidant activity. IC_{50} value of control was 16 µg/ml and on the other hand, IC_{50} value of crude sample was 89 µg/ml which indicated considerable antioxidant activity.

Conclusion

It is apparent that *C. grandis* plant exhibited considerable potential in terms of its biological activities, encompassing antibacterial, antifungal, antioxidant, and cytotoxic properties. Hence, it holds promise as a valuable source for natural prescription. The observed antibacterial activities of both the crude extracts and isolated compounds provide validation for the traditional usage of this plant in treating various bacterial infections. Notably, this study represents the first report on the biological activity of *C. grandis* stem bark in Bangladesh. Consequently, further investigation is warranted to establish this plant as a reliable and efficacious reservoir of natural medicine.

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