Analysis of Proximate composition, Phytochemical profiling, Antimicrobial and Antioxidant activity of methanolic extracts of *Diospyros melanoxylon* Roxb. fruits

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Abstract

D. melanoxylon displays exceptional qualities as a naturally occurring antioxidant and antimicrobial agent. The investigation focuses on phytochemical profiles, proximate composition, antioxidant and antimicrobial activities of methanolic extract of Diospyros melanoxylon fruits. Methanolic extract of the fruits are processed for the determination of total flavonoid and total phenolic content, proximate composition, antioxidant and antimicrobial activity. Different biochemical methods are used for performing these experiment. The determination of total flavonoid content (TFC) and total phenolic content (TPC) is 40.6±4.1µg/mg and 211±5.03 µg/mg respectively. The amount of β-carotene and lycopene is 0.0924± 0.01µg/mg and 0.035± 0.005µg/mg respectively. The amount of total moisture content, total protein content, total carbohydrate content, total fibre content and total lipid content is 85.45± 0.37 g/100g, 6.81 ±0.29 g/100g, 25.78± 0.37 g/100g, 3.13± 0.18 g/100g and 2.39 ±0.04 g/100g, respectively. The IC_{50} values of ABTS and DPPH are 74.65 ±4.38 µg/ml and 59.14 ±1.08 µg/ml respectively. Further, antibacterial activity is studied by the agar well diffusion method using two different bacteria Pseudomonas aeruginosa (MTCC 741) and Staphylococcus aureus (MTCC 87). Antifungal activity is studied by the disc diffusion method against the fungi Candida albicans (MTCC 227).The MIC values of Staphylococcus aureus and Pseudomonas aeruginosa are0.45 mg/ml and 0.30 mg/ml respectively. Fruit extract does not showed antifungal activity. Conducted LC/MS analysis indicated the existence of 7 compounds. Amongst them main is Vallinic acid. Thus, the fruits of Diospyros melanoxylon can be successfully used as constituent in health to reduce oxidative stress.

Keywords: Antibacterial, Antifungal, Antioxidant, *Diospyros melanoxylon*, LC MS, Phytochemicals.

1. Introduction

The genus *Diospyros* is a member of the Ebenaceae family has numerous uses. Different plant parts are utilized as remedies in various folk healing practices, which include therapy for haemorrhage, incontinence, insomnia, hiccoughs, diarrhoea etc. The remedial potential of medicinal plants is naturally associated with their phenolic content, flavonoid content and they play a significant role to reduce oxidative stress [1]. The generic name originates from the Greek word "Dios" which means divine and "Pyros" that means fruits [2]. It is well known for its 'beedi' making leaves worldwide [3].It is

usually known as Kend, Kendu, or Tendu. For healing of scabies and old wounds, leaves and fruits are used in stomach disorders [4]. Supriya and Growther [5] reported that plant extracts and their compounds have strong antioxidant and antimicrobial activity. Based on these results, *D. melanoxylon* appears to be an excellent natural antioxidant and antimicrobial agent and are used in the treatment of various diseases in human being. Kashyap et al. [6] reported that antimicrobial activity, antifungal activity, analgesic activity, anti-diabetic activity, anti-inflammatory activity, and wound healing activity are observed in different plant parts of *Diospyros melanoxylon.* Sailakshmi [7] observed that hydro methanolic extracts of *D. melanoxylon* exhibited heightened antioxidant potency.

An endeavour has been undertaken to explore the photochemical, proximate composition, antioxidant, and antimicrobial properties against certain fungal and bacterial pathogens, of *Diospyros melanoxylon* fruits.

2. Materials and methods

Authentication

The sample is identified by Botanical Survey of India, Howrah with authenticated specimen number VU/ BM-02 for *Diospyros melanoxylon* Roxb.

Collection of samples

Fruits of *D. melanoxylon* are collected from Jhargram forests. Collected plant materials are cleaned frequently with tap water and also in distilled water to eliminate the dust, and shade dried at room temperature for 10- 15 days. The fruit pulp is crushed into fine powder by using a mixer grinder. Dried fine powder is stored at 4℃ for further experiment.

Preparation of plant extracts

Powered plant materials are taken in conical flasks and macerated in methanol (1:10 w/v) and stirred continuously for 72 hours on a shaker. The samples are filtered by using filter paper. Then the extracts are evaporated utilizing a rotary evaporator under reduced pressure and kept at 4°C in airtight containers for future applications.

Phytochemical analysis

Determination of Total Phenolic Content (TPC): The total phenolic content is estimated by the method of Phuyal et al.[8]. Gallic acid is utilized as standard. Optical density (OD) is measured at 760 nm spectrophotometrically. The total phenolic content is assessed as µg in Gallic acid equivalents (GAE) per mg of sample.

Determination of Total Flavonoid Content (TFC): The total flavonoid content is estimated by the method of Phuyal et al.[8]. Quercetin is utilized as standard. Optical density (OD) is measured at 510 nm spectrophotometrically. The total flavonoid content is evaluated as µg in quercetin equivalents (QE) per mg of sample.

Determination of β-carotene and Lycopene content: β-carotene and lycopene contents are estimated as per the protocol of Nagata and Yamashita [9]. 0.1 mL of the extract

undergoes vigorous shaking with 10 mL of hexane: acetone mixture (6:4) for 2 mins and immediate absorbance is measured at 663 nm, 453 nm, and 505 nm wavelengths respectively.

β-carotene and lycopene content are calculated as per the following formula:

$$\beta\text{-carotene } (\mu g/mg) = 0.216\times A_{663} - 0.304\times A_{505} + 0.452\times A_{453}$$

$$\text{Lycopene } (\mu g/mg) = 0.0458\times A_{663} + 0.373\times A_{505} - 0.0806\times A_{453}$$

Analysis by LC/MS: The isolation of photochemical is conducted utilizing a LC-MS system (Shimadzu, Japan LC-MS 2020)assembled with an electro spray ionization (ESI) .The separation of chromatogram is executed utilizing an AQUASIL C18 analytical column (150 mm × 3 mm × 3 mm), maintained at 40^0 C temperature. The mobile phase comprised of 0.1% formic acid in methanol (solvent B) and water(solvent A) at a flow rate of 0.4 mL/min. The elution commences with 70% A/10% B from 0-30 min, 90% B from 30-45 min, from 45-55 min 100% B, from 55-60 min 90% A/10% B. Chromatograms are acquired using a photodiode array detector that is previously set to 350 nm. 10 all volume is injected and peaks are observed at 250 nm. By comparing the Rat (retention time) and the UV spectra with those of authentic standard, peak identification is achieved of fraction phenolic chromatogram. Mass spectra are operated in Multiple Reaction Monitoring (MRM) mode and data are collected. The compounds are analyzed using specific negative ionization modes (m/z[M-H]-) [10].

Proximate composition

The moisture content is assessed as per the method of Thief [11].Total carbohydrate content is determined according to the method of Sadasivam and Manickam[12] with minor changes. The amount of total carbohydrates is estimated as per the DNS method [13].The amount of protein content is estimated by the method of Lowry et al. [14].Total lipid is determined as per the method of Itoh and Kaneko [15].Crude fibre is determined as per the method of Maynard [16].

Antioxidant activity

DPPH assay: The antioxidant potential of fruit extract involves measuring the discoloration of DPPH. Various concentrations of samples are prepared. As a positive control Ascorbic acid is utilized. After incubation, OD value is measured at 517nm spectrophotometrically against the blank. The IC_{50} value is computed to indicate the concentration of the extract where 50% of the DPPH radical was effectively scavenged [17].

$$\text{DPPH radical scavenging (\%)} = [(Abs_{blank} - Abs_{sample}) / Abs_{blank}] \times 100$$

ABTS assay: ABTS assay is determined by Re et al. [18] method. As a standard Trolox is utilized. From the calibration curve, the IC_{50} value is determined

$$\text{ABTS radical scavenging (\%)} = [(Abs_{blank} - Abs_{sample}) / Abs_{blank}] \times 100$$

In vitro antibacterial activity

The antibacterial activity is assessed by agar well diffusion method. Approximately 15-20 ml nutrient agar is poured onto Petri plates and allowed to solidify. To create four wells in the Petri plates, a sterilized cork borer is used. Standardized inoculums of test organisms

(*Pseudomonas* MTCC 741and *Staphylococcus aureus* MTCC 87) are spread over nutrient agar using an L-shaped sterile spreader. Different concentrations of fruit extract are utilized to assess the antibacterial effect. As a positive control Ampicillin is utilized and as a negative control DMSO (20%) is utilized. In an incubator, the Petri plates are placed for 24 hours at 37°C for optimal growth conditions. After incubation, the diameter of zone of inhibition is calculated in millimetres. Minimum inhibitory concentration (MIC) is determined using the broth dilution method [19].

In vitro antifungal activity

Candida albicans (MTCC 227) is utilized for determination of the antifungal activity by disc diffusion assay . Approximately 15-20 ml nutrient agar is poured onto Petri plates and left to solidify. Five days inoculated fungus (10^5 CFU/ml) is swabbed using a sterile swab. Clotrimazole (30 µg/ml) is used as antifungal agent. In an incubator, the Petri plates are incubated for 48 to 72 hours. After incubation, the diameter of zone of inhibition is calculated in millimetres. Minimum inhibitory concentration (MIC) is determined using the broth dilution method [20].

Statistical analysis

All experimented results are presented as mean ± standard deviation (SD) and statistical calculations are done using Microsoft ® Office Excel (Microsoft®, USA). Results are compared using ANOVA to assess variances among samples, with significance levels set at $p < 0.05$ and $p < 0.001$, indicating statistical significance.

3. Result

Phytochemical screening

The methanolic extract exhibited high phenolic (211±5.03µg/mg) and flavonoid (40.6±5.03µg/mg) content. However, the amount of β-carotene (0.0924±0.01µg/mg) and lycopene(0.035±0.005) are very low. The result is shown in Table 1.

Table 1. Phytochemical analysis of methanolic extracts of D. melanoxylon fruits

Total Phenol (µg/mg)	Total Flavonoid(µg/mg)	β-carotene (µg/mg)	Lycopene (µg/mg)
211±5.03	40.6±4.1	0.0924±0.01	0.035±0.005

Analysis of Proximate composition

Moisture content of the fruit extract is very high that is 85.45±0.37 (g/100g). The amount of total carbohydrate content (25.78±0.37 g/100g) is also satisfactory. The amount of protein content, lipid content and crude fibre content are 6.81±0.29 (g/100g), 2.39±0.04 (g/100g) and3.13±0.18 (g/100g) respectively. Result is shown in Table 2.

Table 2. Proximate composition analysis of D. melanoxylon fruits

Moisture content (g/100g)	Carbohydrate content (g/100g)	Protein content (g/100g)	Lipid content (g/100g)	Crude fibre content (g/100g)
85.45 ±0.37	25.78±0.37	6.81±0.29	2.39±0.04	3.13±0.18

Antioxidant activity

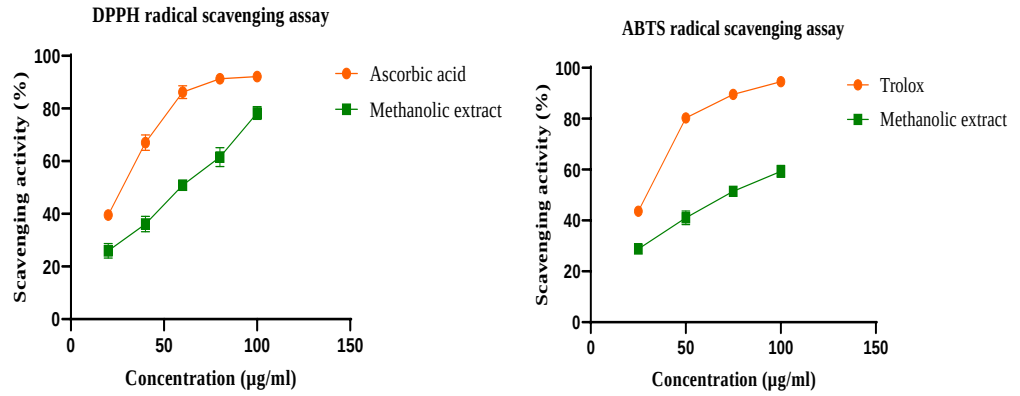
Methanolic extract displayed high antioxidant potential. The IC_{50} value of DPPH is 59.14±1.08 (µg/ml) and ABTS is 74.65±4.38 (µg/ml). Result is shown in Table 3 and the graph is shown in Figure 1.

Table 3. Antioxidant activity of methanolic extracts of D. melanoxylon fruits

Antioxidant activity	Plant extract	Standard
DPPH radical scavenging assay (IC_{50}=µg extract/ml)	59.14±1.08[a]	22.1±0.5[b]
ABTS radical scavenging assay (IC_{50}=µg extract/ml)	74.65±4.38[b]	19.9±1[a]

Result are presented in mean ± SD (n=3)
Different letters in the column show statistically significant differences ($p<0.05$) according to ANOVA.

Figure 1. Antioxidant activity of methanolic extracts of D. melanoxylon fruits



A. DPPH radical Scavenging assay

B. ABTS radical Scavenging assay

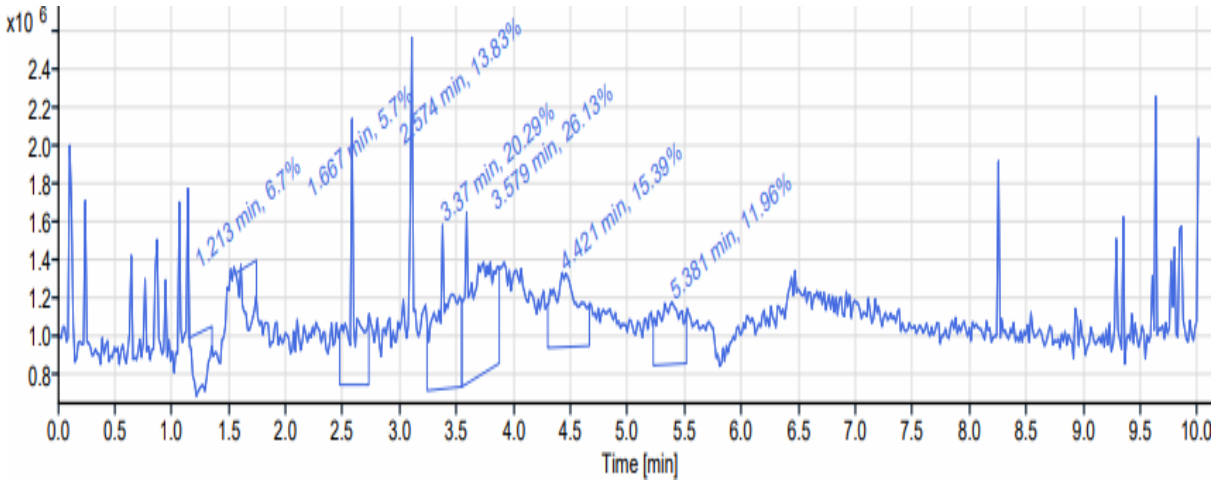
Liquid chromatography and mass spectrometry (LC/MS)

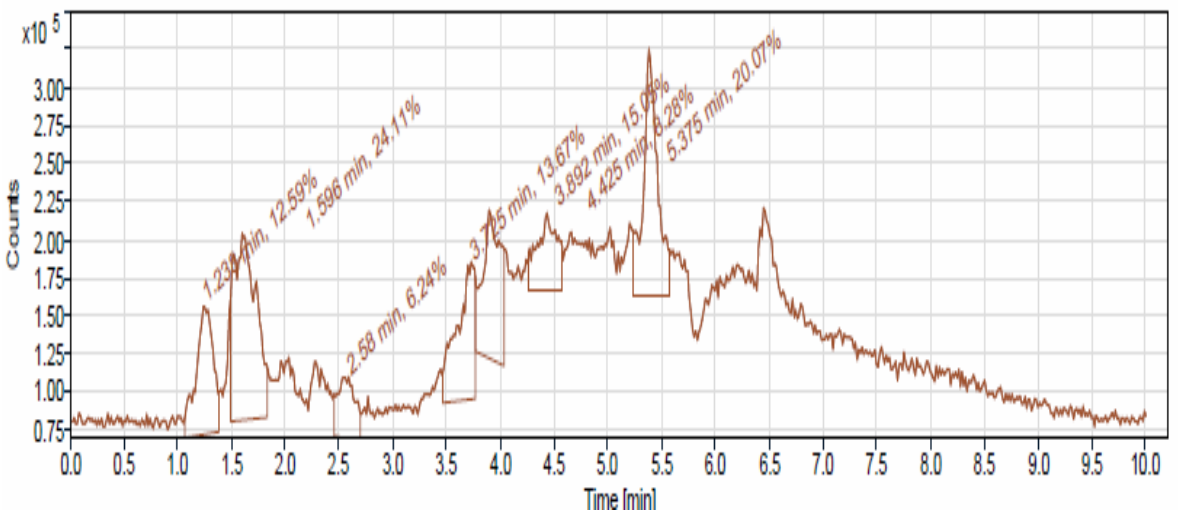
The LC/MS analysis displayed 7 phenolic compounds such as Citronellol (Rt = 1.21 min and area 6.70%), Capsidiol (Rt =1.66 min and area 5.70%),Vanillin acid (Rt = 2.57 min and area 13.83%), Myristicin (Rt =5.38 min and area 11.96%), Carnosic acid (Rt = 4.42 mined area 15.39%),Piceatannol 3-O-glucoside (Rt = 1.59 min and area 24.11%), Catechin-3-O-gallate (Rt= 3.72 min and area 13.67%) on the basis of antioxidant and antimicrobial activity. Presence of compounds and their pharmacological property are shown in Table 4. The graph is shown in Figure 2.

Table 4. Presence of compounds and their pharmacological property

Peak	Name of the compounds	Molecular formula	RT (min)	Area (%)	Pharmacological property	Reference
1.	Citronellol	$C_{10}H_{20}O$	1.213	6.70	Antioxidant	[21]
2.	Capsidiol	$C_{15}H_{24}O_2$	1.667	5.70	Antifungal	[22]
3.	Vanillic acid	$C_8H_8O_4$	2.574	13.83	Antioxidant, antibacterial	[23]
4.	Carnosic acid	$C_{20}H_{28}O_4$	4.421	15.39	Antioxidant, antimicrobial activity	[24], [25]
5.	Myristicin	$C_{11}H_{12}O_3$	5.381	11.96	Antioxidant, antimicrobial activity	[26], [27]
6.	Piceatannol 3-O-glucoside	$C_{20}H_{22}O_9$	1.596	24.11	Antioxidant activity	[28]
7.	Catechin-3-O-gallate	$C_{22}H_{18}O_{10}$	3.725	13.67	Antioxidant, antimicrobial activity	[29]

Figure 2. LC/MS chromatogram of methanolic extract from D. melanoxylon.





Antimicrobial activities of methanolic extracts of D. melanoxylon fruits

The methanolic extract hindered the growth of the Gram positive bacteria *Staphylococcus aureus* (MTCC 87) at 0.45 mg/ml and the Gram negative bacteria *Pseudomonas* (MTCC 741) at 0.3 mg/ml. The zone of inhibition and minimum inhibitory concentration (MIC) are shown in Table 5 and Table 6 respectively. The inhibition zone is displayed in Figure 3.

Table 5. Antimicrobial activity of methanolic extracts of D. melanoxylon fruits

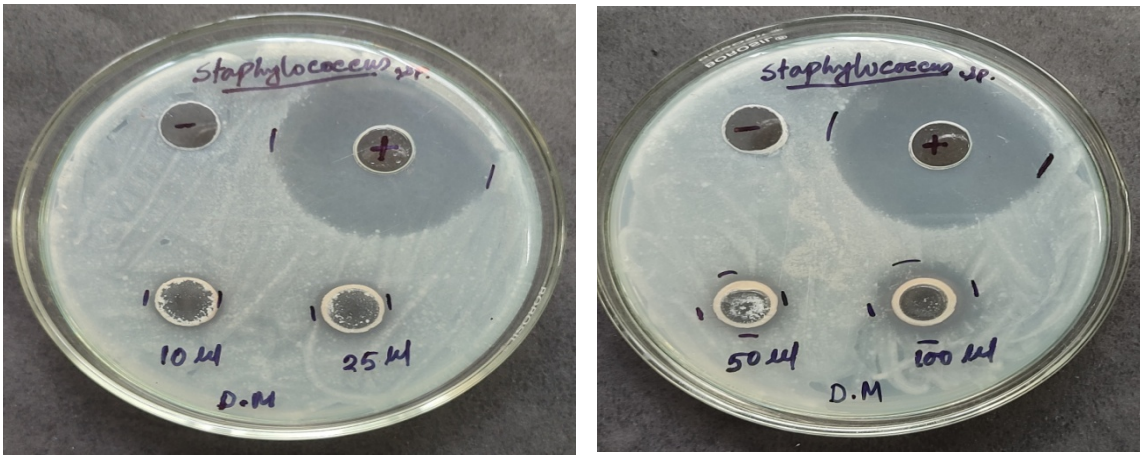
Test Organism	**Cone. (**µg/ml)	**Zone of Inhibition (ZOI) (mm)**		
		Extract	**Positive control**	**Negative control**
Gram Positive bacteria				
Staphylococcus aureus (MTCC 87)	10	11	40	No Zone
	25	12		
	50	14		
	100	20		
Gram Negative bacteria				
Pseudomonas aeruginosa (MTCC 741)	10	13	40	No Zone
	25	14.5		
	50	16		
	100	18		
Fungal strain				
Candida albicans(MTCC 227)	50	No Zone	14	No Zone
	100	No Zone		
	150	No Zone		

Table 6. Determination of Minimum Inhibitory Concentration (MIC)

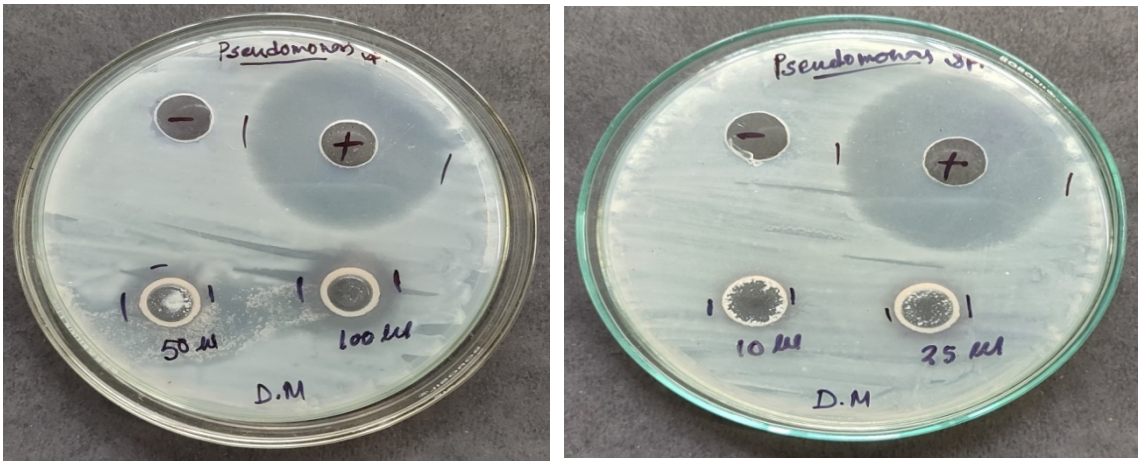
Test Organism	Minimum Inhibitory Concentration (MIC)	
	Extract (mg/ml)	Ampicillin (mg/ml)
Staphylococcus aureus (MTCC 87)	0.45[a]	0.1
Pseudomonas aeruginosa (MTCC 741)	0.3[b]	0.1

Result are presented in mean ± SD (n=3)
Different letters in the column show statistically significant differences ($p<0.05$) according to ANOVA.

Figure 3. Antimicrobial activity of methanolic extracts of D. melanoxylon fruits



Antibacterial activity shown against the Gram positive bacteria *Staphylococcus aureus*. '-' denotes negative control (DMSO); '+' denotes positive control (Ampicillin)



Antibacterial activity shown against the Gram negative bacteria *Pseudomonas aeruginosa*. '-' denotes negative control (DMSO); '+' denotes positive control (Ampicillin)

No antifungal activity shown by the fruit extract against the fungi *Candida albicans*.

Antifungal activity shown by the Standard against the fungi *Candida albicans*.

4. Discussion

In plant, the existence of chemical constituents is responsible for their pharmacological properties. Phenolic compounds can scavenge and eliminate free radicals [30].Methanolic extract of the fruit *D. melanoxylon* exhibited high phenolic content (211±5.03 µg/mg) and high flavonoid content (40.6±4.1 µg/mg) respectively. However, the quantity of β-carotene (0.0924 ±0.01 µg/mg) and lycopene(0.035±0.005 µg/mg) is very low. The IC_{50} value of DPPH of the fruit extract (59.14±1.08 µg/ml) is lower than the fruit *Gardenia latifolia* (65.82 µg/ml) [31] and the IC_{50} value of ABTS (74.65±4.38 µg/ml) is slightly higher than the *Ziziphus nummularia* fruit (66.32 ±0.73 µg/ml) reported by Nisaruddin et al. [32]. The proximate composition is also satisfactory. The moisture content (85.45 ±0.37g/100g) is considerably higher than the fruit of *Carissa carandas* (79.37 ±0.28 g/100g) [33]. The quantity of total carbohydrate content (25.78±0.37 g/100g) is very much higher than the *Rourea* fruit (0.90±0.03 g/100g) [34]. The amounts of total protein content(6.81±0.29 g/100g), total lipid content(2.69±0.04 g/100g) and crude fibre(3.13±0.18 g/100g) are higher than the Banana fruit (4.4 ±0.9 g/100g), Papaya fruit (1.4± 0.1 g/100g) and watermelon (2.8± 0.2 g/100g) respectively [35]. The fruit extract revealed high antibacterial activity. The MIC value against the Gram-negative bacteria *Pseudomonas* sp. is 0.3 (mg/ml) and Gram-positive bacteria *Staphylococcus* sp. is 0.45 (mg/ml) respectively, which showed strong activity than the fruit extract of *Momordica cochinchinens* is reported by Tinrat et al. [36]. They showed that the MIC value of *Pseudomonas* sp. is ranging from 50-100 (mg/ml) and *Staphylococcus* sp. is >100 (mg/ml). However, the extract doesn't show any activity against *Candida albicans*. No one has previously reported the presence of Vallinic acid of *Diospyros melanoxylon* fruits. But LC/MS analysis revealed the existence of Vallinic acid in the methanolic extract of *Diospyros melanoxylon* fruits.

5. Conclusion

In the present study, proximate composition, phytochemical profiling, antimicrobial and antioxidant activity are evaluated of methanolic extract of *Diospyros melanoxylon* fruits. Results displayed that fruits of *Diospyros* sp. have a good quantity of nutritional (Carbohydrate, Protein, and Lipid) contents which may be effective as a food supplement other than conventional fruits available in the market. Phytochemical contents are also satisfactory. Consumption of those fruits may help in the prohibition of many disorders such as cardiovascular diseases, diabetes, cancer etc. Antioxidant activity and antimicrobial activity are also satisfactory compared to other fruits. Consumption of these fruits may prevent skin damage, aging, Alzheimer's and Parkinson's disease. Numerous inquiries persist regarding the efficacy of antioxidant supplements in disease prevention. Additional research is imperative before endorsing any supplementation as an official adjuvant therapy.

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Conflict of interest

The Authors declared that there is no conflict of interest.

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