CHAPTER 5

RESULTS

5.1 Phytochemical analysis of *Anzia ornatoides*

5.1.1 Extraction yield

The dry extracts of *A. ornatoides* prepared in the five different solvents showed various colours depending on the chemical components extracted in each solvent (Table 5.1)

Table 5.1. Temperature values for refluxing at Soxhlet, extraction yield and colours of *A. ornatoides* dry extracts using different solvents

Extracts	Temperature (°C)	Yield (%)	Colour after extraction
Hexane	68	0.6	Olive yellow
Diethyl ether	34.6	4.56	Yellowish green
Ethyl acetate	77.1	7.33	Greenish yellow
Methanol	64.7	13.83	Brownish yellow
Water	100	18.26	Reddish brown

5.1.2 Preliminary phytochemical screening of *Anzia ornatoides*

Table 5.2. Preliminary phytochemical screening of *A. ornatoides* with various solvents

Lichen constituent	Name of test	Extracts					Appearance of the colour
		HE	DE	EA	ME	WE	
Alkaloid	Mayer's test	+	−	−	+	+	Creamy white ppt.
	Wagner's test	−	−	−	+	+	Reddish brown ppt.
Carbohydrate	Benedict's test	+	+	+	+	+	Green ppt.

	Fehling's test	+	+	+	–	–	Brick red ppt.
Flavonoid	NaOH test	+	–	–	+	+	Yellow to orange ppt.
	Lead acetate test	+	–	–	+	+	Yellow ppt.
Glycosides	Keller-kilani test	+	+	+	+	+	Brown ring at interphase
	Salkowski's test	+	+	+	+	+	Brown ring at interphase
Phenol	Ferric chloride test	+	–	–	+	+	Black ppt.
	Lead acetate test	+	–	–	+	+	White ppt.
Saponins	Foam test	–	–	–	–	–	No colour formed
	Honey comb test	+	–	–	+	+	Honey comb like froth

(**Note**: HE - Hexane extract, DE - Diethyl ether extract, EA - Ethyl acetate extract, ME - Methanol extract, WE - Water extract, ppt. - Precipitate)

5.1.3 GC-MS analysis

GC-MS was used to analyse the volatile components of *A. ornatoides* to establish their chemical composition (Fig. 5.1). A total of 21 volatile chemicals have been identified (Table 5.3), representing three significant substances viz. benzoic acid 2,4-dihydroxy-3,6-

dimethyl-methyl ester (4.77%), imidazole 2-t-butyl-1,4-dimethyl-5-phenyl (6.32%), and methyloxyolivetol (78.91%).

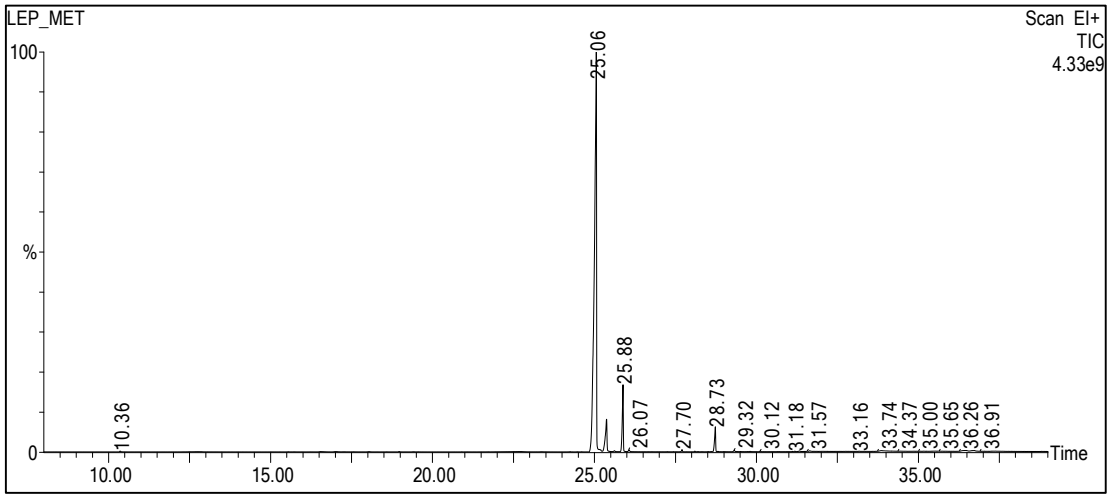

Fig. 5.1. GC-MS chromatograph of the methanol extract of *A. ornatoides* volatile compounds with putative chemical structures of most abundant molecule

Table 5.3. Compounds detected and identified in the methanol extract by GC-MS

Sl No.	Name of the compound	RT (min)	Peak area (%)	M.W.
1	Benzeneacetaldehyde	15.541	0.017	120
2	Phosphonic acid, (p-hydroxyphenyl)-	10.359	0.103	174
3	Hydroquinone	16.542	0.123	110
4	2(1h)-pyridinone, 3-amino-	17.012	0.124	110
5	1-propanamine, 3-(2-methoxy-1-methylethoxy)-	18.032	0.057	147
6	.Alpha.-[2-piperidyl]-2-trifluoromethyl-6,8-dichloro-4-Quinolinemethanol	18.973	0.047	378
7	Methoxyolivetol	25.055	78.916	194
8	Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester	25.37	4.77	196
9	Imidazole, 2-t-butyl-1,4-dimethyl-5-phenyl	25.881	6.324	228
10	1,3-benzenediol, 5-pentyl-	26.066	0.214	180
11	Pyrazole-4-carboxaldehyde, 3-(4-chlorophenyl)-	27.701	0.19	206

12	Pyrazole, 3-(p-chlorophenyl)-5-methyl-	28.096	0.054	192
13	O-anisic acid, 4-hydroxy-6-pentyl-, methyl ester, ester with 2-hydroxy	28.732	2.393	514
14	Octadecanoic acid	29.317	0.179	284
15	2,6,10-dodecatrien-5-one, 1-(2,5-dihydroxy-3-methylphenyl)-3,7,11-trimethyl	30.117	0.057	422
16	Z,Z,Z-8,9-epoxyeicosa-5,11,14-trienoic acid, methyl ester	31.183	0.036	334
17	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1s-(1.alpha.,2.beta.,5.beta.)]	31.318	0.07	156
18	1-methylene-2b-hydroxymethyl-3,3-dimethyl-4b-(3-methylbut-2-enyl)-cy	31.638	0.125	222
19	Alpha.-linolenic acid, tms derivative	33.864	0.506	350
20	2(1h)-benzocyclooctenone, decahydro-10a-methyl-, trans-	36.42	0.299	194
21	2-oxo-2,3-dihydro-1h-imidazole-4-carbonitrile	36.675	0.206	109

5.2 Antioxidant activity of *Anzia ornatoides*

5.2.1 Total phenolic contents

The total phenolic compounds of the extracts were determined as the gallic acid equivalent using an equation derived from a standard gallic acid graph, $y = 0.0064x + 0.024$, $R^2 = 0.9961$ (Fig. 5.2A). The findings of the study indicated the phenolic compounds in the extracts ranged from 2.93±0.13 mg GAE g^{-1}DW to 8.27±0.13 mg GAE g^{-1}DW (Fig. 5.2B). The methanol extract of *A. ornatoides* had the maximum phenolic concentration, whereas the diethyl ether extract had the lowest phenolic content. The increasing order of the variations was methanol > water > ethyl acetate > hexane > diethyl ether. At $P < 0.01$, a statistically significant difference was observed (Table 5.4).

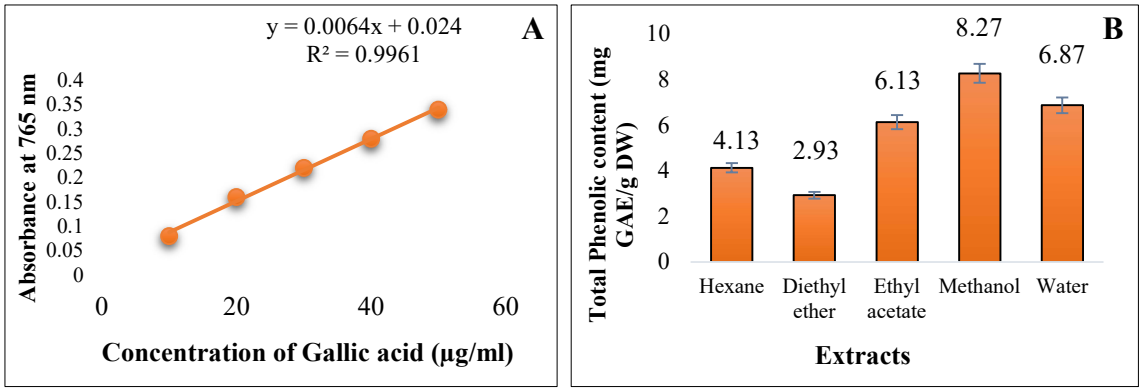

Fig. 5.2. A - Standard graph of Gallic acid curve, B - Total phenolic contents (mg GAE/g DW) of different extracts

5.2.2 Total flavonoid contents

An equation derived from a standard graph, y = 0.009x – 0.046, R^2 = 0.9992, was used to quantify the flavonoid molecules as the quercetin equivalent (Fig. 5.3A). Flavonoid contents of extracts ranged from 1.53±0.07 mg QE g^{-1} to 2.00±0.00 mg QE g^{-1}DW. The methanol extract exhibited the highest flavonoid content, whereas diethyl ether displayed the lowest flavonoid content, as noted in figure 5.3B. The increased variation showed in the order of methanol > hexane > water > ethyl acetate > diethyl ether. A statistically significant difference was seen at $P < 0.01$ (Table 5.4).

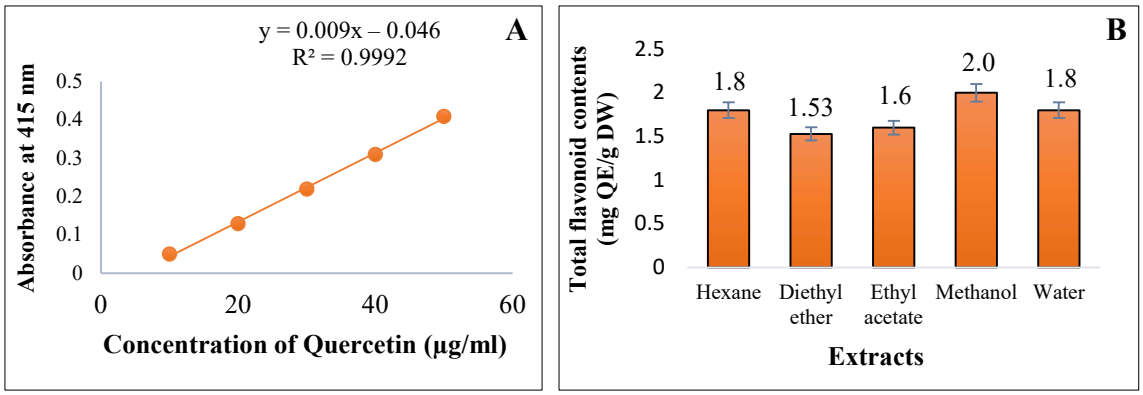

Fig. 5.3. A - Standard graph of Quercetin curve, B - Total flavonoid contents (mg QE/g DW) of different extracts

5.2.3 Phosphomolybdenum assay

Using an equation derived from a standard ascorbic acid graph, y = 0.0031x – 0.011, R^2 = 0.9981, the total antioxidant compounds were evaluated as the ascorbic acid equivalent (Fig. 5.4A). The findings demonstrated the evaluated antioxidant chemicals ranged from 2.73±0.67 mg AAE g^{-1}DW to 13.13±0.67 mg AAE g^{-1}DW (Fig. 5.4B). The increased variation presented in the order of water > methanol > hexane > diethyl ether > ethyl acetate. At P < 0.01, there was a statistically significant difference (Table 5.4).

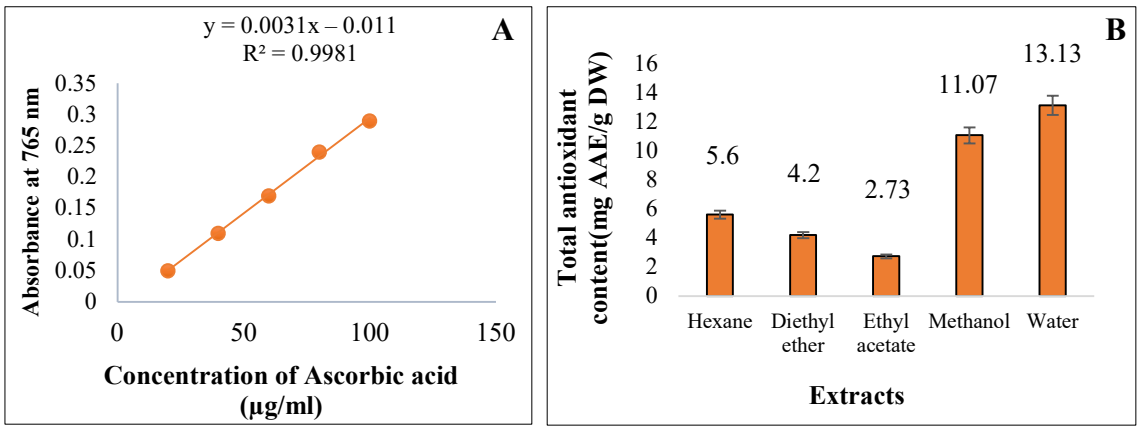

Fig. 5.4. A - Standard graph of Ascorbic acid, B - Total antioxidant compounds by phosphomolybdenum method (mg AAE/g DW) of different extracts

5.2.4 Ferric-reducing antioxidant power assay

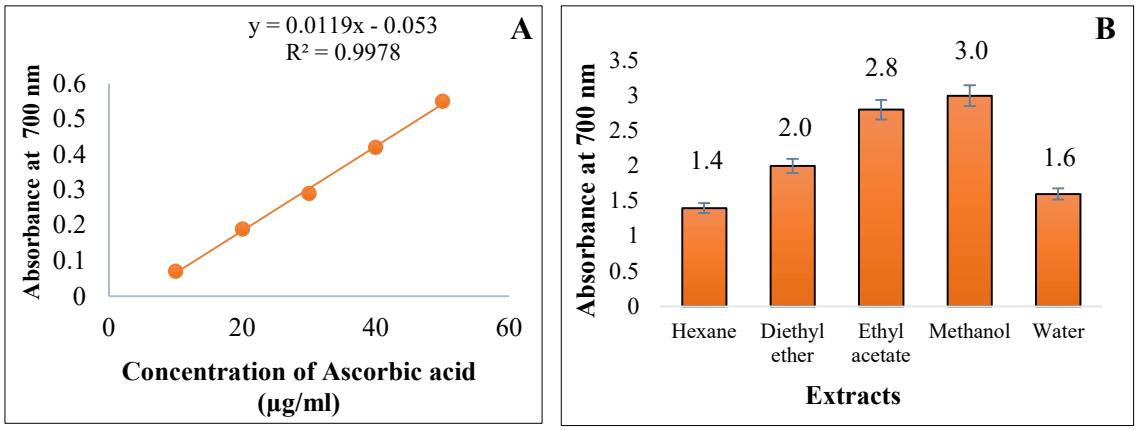

Fig. 5.5. A - Standard graph of Ascorbic acid, B - Ferric reducing antioxidant power (mg AAE/g DW) of different extracts

An equation derived from a standard ascorbic acid graph, y = 0.0119x – 0.053 R^2 = 0.9978, was used to assess the FRAP activity as the ascorbic acid equivalent (Fig. 5.5A).

In Fig. 5.5B, the reducing power assay of the extracts are summarized. Greater reducing power is indicated by higher absorbance of the reaction mixtures. A range of 1.4 to 3.0 was seen in the absorbance readings. Methanol demonstrated the greatest reducing power among the studied extracts, whereas hexane displayed the lowest. The reducing power of extracts increased in the following order: methanol > ethyl acetate > diethyl ether > water > hexane. At $P < 0.01$, a statistically significant difference was observed (Table 5.4).

Table 5.4. Results of four assays among five different extracts by Kruskal-Wallis test

Assays	χ^2	df	P-value	Significance
TPC	13.57	4	0.009	**
TFC	13.66	4	0.008	**
Phosphomolybdenum assay	13.77	4	0.008	**
FRAP	14	4	0.007	**

5.2.5 Scavenging DPPH radicals

The percentage of scavenging DPPH radicals in each of the extracts are presented in Table 5.5. The IC50 values of extracts for DPPH scavenging are shown in Fig. 5.6B. There was a statistically significant difference between extracts and concentration at $p < 0.05$, $P < 0.01$ (Table 5.6A & 5.6B). The method has been employed for the evaluation of free radical scavenging activity of several natural product extracts (Mbaoji and Nweze, 2020). Ascorbic acid was used as a standard (Fig. 5.6A).

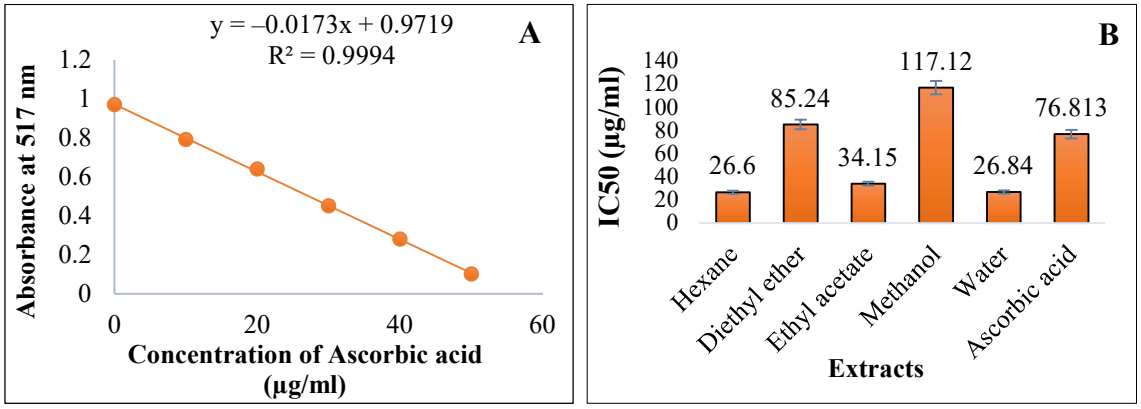

Fig. 5.6. A - Standard graph of Ascorbic acid, B - IC50, scavenging DPPH radical between extracts

Table 5.5. Percentage scavenging rate of DPPH free radicals by extracts

Conc. (µg/ml)	Extracts				
	Hexane	Diethyl ether	Ethyl acetate	Methanol	Water
10	10.30±1.00	11.80±0.85	15.44±0.16	11.65±0.73	5.60±0.27
20	10.92±0.74	14.86±1.18	21.81±0.84	18.04±0.54	10.77±0.31
30	12.17±0.16	18.80±0.32	27.19±0.11	22.17±0.40	14.01±0.26
40	12.51±0.12	24.13±0.57	30.71±0.91	28.02±0.15	17.16±0.05
50	13.06±0.21	29.42±0.03	33.85±0.38	33.27±1.65	18.47±0.11

The data is represented in percentage ± SD (n = 3).

Table 5.6A. DPPH assay among the extracts in each concentration by Kruskal-Wallis test

Conc. (µg/ml)	χ^2	df	P-value	Significance
10	11.49	4	0.02	*
20	12.92	4	0.01	**
30	13.5	4	0.009	**
40	13.54	4	0.008	**
50	12.92	4	0.01	**

Table 5.6B. DPPH assay among the concentration in each extract by Kruskal-Wallis test

Extracts	χ^2	df	P-value	Significance
Hexane	10.12	4	0.04	*
Diethyl ether	13.26	4	0.01	**
Ethyl acetate	13.50	4	0.009	**
Methanol	13.55	4	0.008	**
Water	13.55	4	0.008	**

5.2.6 ABTS assay

The percentage ABTS scavenging activities of extracts are shown in Table 5.7. There was a statistically significant difference between extracts and concentration at $P < 0.01$ (Table 5.8A & 5.8B). The IC50 values for the extracts of ABTS scavenging capabilities are shown in Fig. 5.7B. Hexane extract showed the highest IC50 value, while ethyl acetate exhibited the lowest. Trolox was used as a standard (Fig. 5.7A).

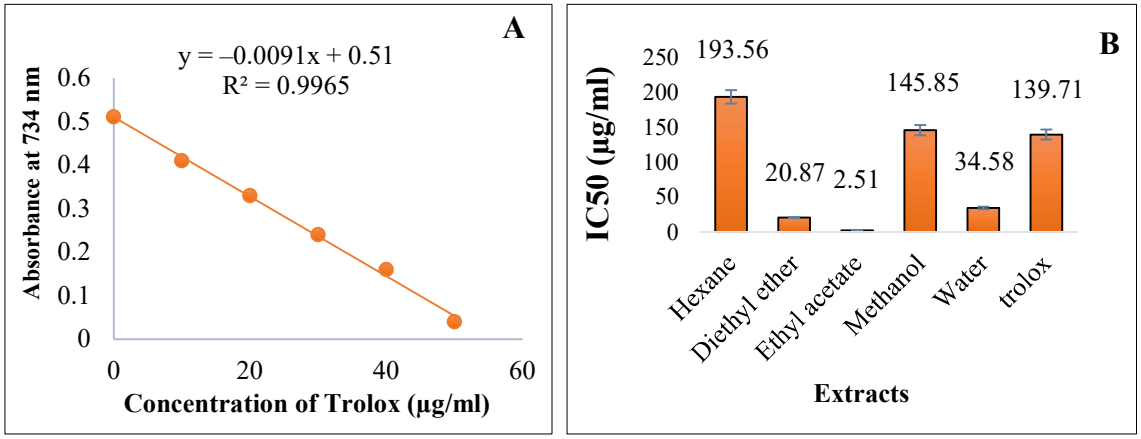

Fig. 5.7. A - Standard graph of Trolox, B - IC50, ABTS assay between extracts

Table 5.7. Percentage scavenging rate of ABTS radicals by extracts

Conc. (µg/ml)	Extracts				
	Hexane	Diethyl ether	Ethyl acetate	Methanol	Water
10	9.85±1.32	15.00±1.32	42.98±0.46	6.33±1.40	6.85±0.13
20	21.98±1.74	32.48±3.09	58.25±0.65	15.78±0.43	9.72±0.06
30	33.33±0.57	43.77±1.14	59.30±0.11	24.33±0.34	12.65±0.07
40	42.99±0.46	51.73±0.75	60.73±0.17	32.36±0.28	26.74±0.46
50	57.66±0.52	53.69±1.13	65.56±0.69	41.62±0.13	30.46±1.94

The data is represented in percentage ± SD (n = 3).

Table 5.8A. ABTS assay among the extracts in each concentration by Kruskal-Wallis test

Conc. (µg/ml)	χ^2	df	P-value	Significance
10	12.26	4	0.01	**

20	13.55	4	0.009	**
30	13.52	4	0.008	**
40	13.50	4	0.009	**
50	13.55	4	0.008	**

Table 5.8B. ABTS assay among the concentration in each extract by Kruskal-Wallis test

Extracts	χ^2	df	P-value	Significance
Hexane	13.52	4	0.009	**
Diethyl ether	13.23	4	0.01	**
Ethyl acetate	13.55	4	0.008	**
Methanol	13.52	4	0.008	**
Water	13.28	4	0.009	**

5.2.7 Inhibitory activity towards lipid peroxidation

The findings of the extract's inhibition of lipid peroxidation activity are compiled in Table 5.9. At $P < 0.05$ and $P < 0.01$, there was a statistically significant difference between the concentration and extracts (Table 5.10A & 5.10B). The IC50 values of extracts for inhibiting lipid peroxidation are shown in Fig. 5.8B. Hexane extract had the highest IC50 value while water extract had the lowest. Ascorbic acid was used as a standard (Fig. 5.8A).

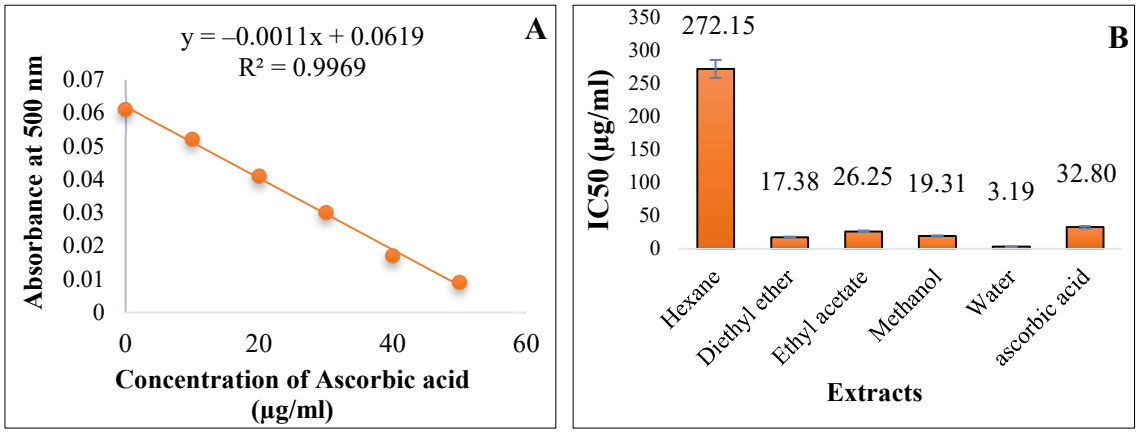

Fig. 5.8. A - Standard graph of Ascorbic acid, B - IC50, Inhibitory activity towards lipid peroxidation between extracts

Table 5.9. Percentage scavenging rate of lipid peroxidation radicals by extracts

Conc. (µg/ml)	Extracts				
	Hexane	Diethyl ether	Ethyl acetate	Methanol	Water
10	5.38±0.53	24.73±0.54	11.83±0.53	25.27±0.54	31.19±0.53
20	39.25±1.07	45.16±2.46	23.12±1.94	49.46±1.42	60.21±1.08
30	47.85±1.08	56.45±0.93	48.93±0.54	62.37±2.99	63.44±0.54
40	57.52±0.54	60.22±0.54	57.52±0.54	73.65±0.54	66.13±0.00
50	80.65±0.93	62.90±0.93	66.13±1.61	77.42±5.30	72.58±0.00

The data is represented in percentage ± SD (n = 3).

Table 5.10A. Lipid peroxidation assay among the extracts in each concentration by Kruskal-Wallis test

Conc. (µg/ml)	χ^2	df	P-value	Significance
10	13.16	4	0.01	**
20	12.95	4	0.01	**
30	11.79	4	0.02	*
40	13.23	4	0.01	*
50	13.15	4	0.01	**

Table 5.10B. Lipid peroxidation assay among the concentration in each extract by Kruskal-Wallis test

Extracts	χ^2	df	P-value	Significance
Hexane	13.60	4	0.008	**
Diethyl ether	13.43	4	0.009	**
Ethyl acetate	13.60	4	0.008	**
Methanol	13.55	4	0.008	**
Water	13.77	4	0.008	**

5.3 Discussion

Phytochemical research is crucial when it comes to finding new sources of substances that have therapeutic and scientific value. The data obtained from the study demonstrated that this lichen thalli has some kind of medicinally valuable chemical. The lichen *A. ornatoides* contained alkaloids, carbohydrates, flavonoids, glycosides, phenols, and saponins, as shown by our current analysis. The information regarding the identification and standardisation of secondary metabolites is improved by the current study. It is beneficial to conduct additional research on notable sources of distinctive bioactive chemicals for its therapeutic and pharmacological applications. These substances might have the potential to be safe, environmentally benign substitutes for pesticides or fungicides, as well as biodegradable products and a safe option for treating infectious diseases.

The outcome showed that more phenolic chemicals accumulated in polar solvents than in non-polar solvents (Fig. 5.2B). The variation could have been attributed to different solvent polarities (Vyas *et al*., 2015). Hydroxyl groups, which are important components in phenolic substances that contribute to antioxidant activity and act as free radical terminators. These substances are the main chief agents that can donate hydrogen to free radicals, and which can stop the lipid oxidation chain reaction at the first stage. Phenolic contents can scavenge the development of free radicals in the human body due to the presence of their hydroxyl groups (Nur *et al*., 2019).

Flavonoid compounds were also accumulated in polar rather than non-polar solvents (Fig. 5.3B). The most significant naturally occurring phenols are flavonoids, which have a wide range of biological and chemical activities, including the ability to scavenge radicals. It is known to be polyphenolic compounds comprising two phenyl rings linked by a propane bridge, resulting in a characteristic 15-carbon (C6-C3-C6) flavan skeleton (Neilson *et al*., 2017). It can be regarded as a class of phenolic compounds with low molecular weight that can neutralise the free radicals in the human body; the activity depends on the number and location of the OH group, which are related to the ability of these compounds to donate electrons (Vyas *et al*., 2015; Zawawi *et al*., 2021).

As indicated by Fig. 5.4B, the water extract contained the highest concentration of antioxidant compounds, whereas ethyl acetate had the lowest antioxidant compound. The antioxidant compounds can counteract the free radicals that do not have a partner and become unstable with high reactivity (Saroyo and Nur, 2020).

The reducing capacity of compounds might be a significant indicator of its possible antioxidant function. The reducing potential is typically associated with the presence of its reductons, which are created when a hydrogen atom is donated to break the chain of free radicals. The strength of the blue-green resulting solution, which absorbs at 700 nm, indicates the reduction of ferrous ions (Fe^{3+}) to ferric ions (Fe^{2+}). The result shown in Fig. 5.5B, demonstrates the ferric reducing power activity of extracts is due to the presence of their polyphenol components, which may function similarly to reductons by giving electrons and reacting with free radicals to transform them into more stable compounds and stop free radical chain reactions. In this assay, there is an electron transfer mechanism present that could affect the activity of phenolic compounds present in the extracts (Dobros *et al*., 2022).

This DPPH assay is used for the preliminary test to determine the reactivity of the test extract compounds. When the extract compound reduces the stable free radical, which produces a purple colour and a strong absorption maximum band at 517 nm, the absorbance decreases and the colour changes from purple to yellow. It has been reported that the antioxidant activity of this lichen might be attributed to the presence of the hydroxyl functional group of phenolic compounds having redox properties (Borkataky *et al*., 2013).

ABTS have been also operated for the evaluation of free radical scavenging activity of extracts using trolox as a standard, which stabilizes the free radicals through proton donors and provides lipophilic and hydrophilic compounds through proton donors and provides lipophilic and hydrophilic compounds (Chohra *et al*., 2020).

Table 5.9 illustrates the concentration-dependent prevention of lipid peroxidation by the extracts at varying concentrations, which became statistically significant at higher doses. Lipid peroxidation method is a toxicological process, which is responsible for the excessive production of reactive oxygen species causes modification of lipoprotein, DNA sequences and protein (Ananthi *et al*., 2015).

Lichen extracts showed a reasonably high level of antioxidant activity that is connected to flavonoid and phenolic components, are able to neutralise free radicals including superoxide, singlet oxygen, and hydroxyl radical, which makes them considerably more effective at scavenging radicals. The discovery of this work provides strong evidence that *A. ornatoides* extracts are a potent radical scavenger and contain a variety of naturally occurring antioxidants and active bioactive chemicals with desired therapeutic potential.