

HIGH RESOLUTION LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY ANALYSIS (Q-TOF-MS) AND FREE RADICAL SCAVENGING ACTIVITY OF EXTRACT OF *AZADIRACHTA INDICA* LEAVES

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ABSTRACT

An indigenous tree to the entire Indian subcontinent, *Azadirachta indica* (neem), a member of the Meliaceae family, is known for its ability to promote good health due to the abundance of antioxidants and flavonoids. The present study is focused on investigating the phytochemical composition of *Azadirachta indica* aqueous leaf extract using the high resolution liquid chromatography-mass spectrometry (HRLC-MS) method as well as the free radical scavenging potency that makes them beneficial for various therapeutic strategies. The first polyphenolic flavonoid quercetin and cynaroside were isolated from freshly harvested neem leaves and were recognized to have anti-oxidant, anti-inflammatory, antifungal and antibacterial properties. Other important phytoconstituents having similar medicinal properties such as Aurachin D, Gentisic acid, Allivicin, Lactapiperanol C, Bruceantin, Caulerpin, Copalliferol B were identified by HPLC-MS. The neem extract's ability to neutralise 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals *in-vitro* was evaluated. The findings of this study showed that leaf extracts of *A. indica* contains a variety of phytochemical compounds with therapeutic potential and can expertly protect the body against oxidative stress brought on by free radicals.

Keywords: Antioxidants, *Azadirachta indica*, Chromatography, Phytochemicals, Spectrometry

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Azadirachta indica, a native of the Indian subcontinent is a member of the "Meliaceae" family (Alzohairy, 2016). It is commonly referred to as the neem tree, is a tropical evergreen tree that is unique to the Indian subcontinent (Aneesa, 2016). The word "neem" derived from the Sanskrit word "nimba" is classified as "Village Pharmacy" or "Devine Tree" or "Nature's Drugstore" or "Medicinal Cabinet" or "Panacea" implying that it can treat all illnesses (Islas *et al.*, 2020). Further, it is worthy to mention that it contains glycoproteins, triterpenes, limonoids, flavonoids, phenols, tannins, nimbins, saponins, catechins, azadirachtin, and gallic acid are the main phytochemicals found in neem (Gupta *et al.*, 2017). The anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, antigastric ulcer, antifungal, antibacterial, antiviral, antimalarial, and antitumor actions of these substances have been validated by earlier researchers (Srivastava *et al.*, 2020; Shewale, 2022). The medicinal value of neem has been recognized by the US National Academy of Science, which published a report in 1992 entitled, "Neem—A Tree of Solving Global Problem" (Joshi *et al.*, 2010). The golden plant established the fact that polyphenolic compounds in neem possess remarkable antioxidant properties (Vardhan and Sahoo, 2020). It modulates many cellular and molecular pathways, such as free radical scavenging, to exert its potentially beneficial benefits (Heyman *et al.*, 2017). The

flavonoids and their derivatives like cynaroside, quercetin, gentisic acid and maritimetin were found among those screened antioxidant phytochemicals that could serve as a potential source of antioxidants and can be explored as therapeutic agents in free radical-induced diseases. Therefore, taking into account these previous reports that the *A. indica* has huge medical importance and this study was conducted to evaluate in detail phytochemical studies using most advanced phytoanalysis technique, HPLC-MS, for promising identification of the phytochemicals and to analyze antioxidant efficiency in the aqueous extract of *A. indica*.

MATERIAL AND METHOD

Preparation of *Azadirachta indica* leaf extract

Fresh leaves of *A. indica* were collected, washed under running tap water and then with distilled water to eradicate undesirable dirt and other foreign materials. The sample was air dried under shade until no moisture left. Approximately, 20g of *A. indica* leaves were minced properly and soaked overnight in 100 ml of sterile distilled water. The resulting mixture was heated at 60° C for 10 minutes. Subsequently, the mixture was centrifuged at 6000 rpm for 15 minutes and filtered using Whatman No. 1 filter paper. The pH of the extract was maintained at 7.3 and evaporated extract was stored at 4°C for further use (Roy *et al.*, 2017).

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Phytochemical characterization of Aqueous Neem Extract

HPLC–MS analysis for antioxidant and other bioactive components was performed on a TOF/Q-TOF Mass Spectrometer (Agilent Technologies). Data acquisition and processing was accomplished using Agilent 6200 Series.

Specifications of LC-MS method metabolite_ESI_+VE_MS/MS

The neem extract was characterized by HPLC–MS based on the method metabolite_ESI_+VE_MS/MS. This method had been carried out to permit the rapid qualitative analysis of Neem (*Azadirachta indica*) aqueous extract. HPLC–MS analysis of antibacterial and antioxidant components was performed on a TOF/Q-TOF Mass Spectrometer, (Agilent Technologies). Data acquisition and processing were accomplished using Agilent 6200 Series. The HPLC-MS method may tolerate less sample preparation and purification in addition to having great sensitivity, which significantly reduces overall analysis time. The injection volume of 10 μ L, desorption liquid temperature 250°C, Threshold (Abs) 10000, Nebulizer (psig) 35, target (counts/spectrum) 25000.000, Purity Stringency (%) 100.000, Purity Cutoff (%) 30.000 was used. Full mass scan spectra were recorded in the negative ionization mode over the range of m/z 120-1200 (1 scan/s). High Pressure Limit was 200.00 bar, Flow 0.300 mL/min., Draw Speed 100.0 μ L/min, Eject Speed 100.0 μ L/min and Spectrum Range WL from 190 to 640nm. The phytochemicals were identified according to their retention time, pattern of mass spectra and its comparison with the KEGG, METLIN and CAS compound data bases. Compounds were identified by comparison of their retention time and UV and mass spectral data with test substances. According to injected absolute levels, the limit of detection for azadirachtin in extract samples was found to be (about) 1 ng/ml or 10 pg in SIM mode which is approximately 1000-times lower than values quoted in the literature for existing HPLC methods (approximately 200 ng/ml or 10 ng).

DPPH free radical scavenging assay

According to the method described in (Pokhrel *et al.*, 2015), with a small modification, the antioxidant activity of Neem was measured on the basis of the scavenging activity of the stable 2, 2- diphenyl-2-picrylhydrazyl (DPPH) free radical. In brief, 100 μ L AgNPs received 0.1 mM of DPPH in methanol. Each extract was mixed with 3 ml of a methanolic solution containing DPPH radicals (0.2, 0.4, 0.6, 0.8, or 1 mg/mL). At 517 nm, the test tubes' absorbance was measured after 30 minutes of room

temperature incubation. A lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The test solutions were prepared using the same amounts of ascorbic acid as the standard. The difference in absorbance between the test and the control (DPPH in ethanol) was calculated and expressed as % scavenging of DPPH radical. The capability to scavenge the DPPH radical was calculated by using the following standard equation. Scavenging effect (%) = $(1 - A_s/A_c) \times 100$; whereas 'A_s' is the absorbance of the sample at t=0 min and 'A_c' is the absorbance of the control at t=30 min.

RESULT AND DISCUSSION

HPLC- MS analysis

The HPLC- MS analysis revealed several peaks in aqueous extract of *A. indica* (neem tree). The plant has demonstrated numerous health benefits, including immunomodulatory, antimalarial, antifungal, antioxidant, antibacterial, anticarcinogenic, antimutagenic, antiulcer and anti-inflammatory characteristics. Therefore, in this study, the phytochemical constituents determined by high performance liquid chromatography analysis and the antioxidant properties of aqueous extracts of *A. indica* leaves were investigated in both positive and negative ion modes to assess the potential protective benefits of this plant against degenerative reactions induced by free radicals.

From the chromatogram report in Fig. 1(a) and (b), the phytochemicals identified were of pharmacological importance. Saponins, flavonoids and alkaloids were only present in the aqueous extract, while steroids were only present in the ethanol extract (Hikaambo *et al.*, 2022). In Table 1, the phytochemical screening of the *A. indica* leaves revealed the presence of phenolics and tannins in aqueous extract. The LC-MS-based untargeted analysis in Table 2 showed the phytochemical profile of the leaves of *A. indica* in which 117 phytochemicals from different chemical classes were annotated, including organic acids, phenolic acids, flavonoids, and other compounds and in Fig. 2, ESI-MS fingerprint spectra of the above discussed phytochemicals from the leaves of *A. indica* with wide significance were postulated.

Anti-oxidant Activity

The results of the HPLC-MS studies confirm the presence of a wide array of phytoconstituents with antioxidant properties. Antioxidants have an important role in the activation of antioxidant enzymes that play a part in the regulation of damage produced by free radicals/reactive oxygen species. Antioxidants can reduce oxidative damage indirectly by boosting the activity or expression of intracellular antioxidant enzymes or directly

Table 2. Structure and bioactivity of principle phytochemicals identified in the aqueous extracts of the Leaves of *A. indica*

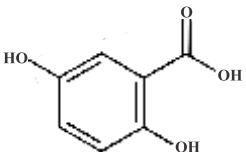
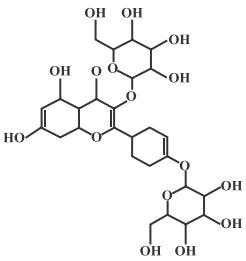
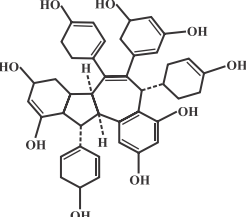
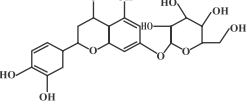
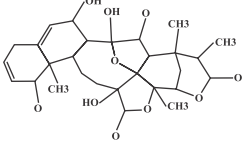
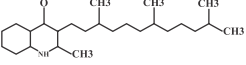
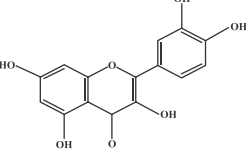
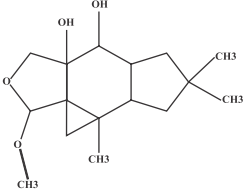
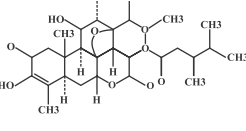
S.No.	Name of compound	Structure	Bioactivity with reference
1	Gentisic acid		anti-inflammatory, antirheumatic and antioxidant (Mardani-Ghahfarokhi and Farhoosh, 2020)
2	Allivcin		anti-bacterial (Geewananda <i>et al.</i> , 1986)
3	Copalliferol B		anti-bacterial (Geewananda <i>et al.</i> , 1986).
4	Cynaroside		antibacterial, antifungal and anticancer activities, antioxidant and anti-inflammatory (Rehfeldt <i>et al.</i> , 2022)
5	Physalin O		anti-inflammatory (Ji <i>et al.</i> , 2012)
6	Aurachin D		antibacterial and anti-inflammatory (Kruth <i>et al.</i> , 2022)
7	Quercetin		antioxidant, antibacterial and antifungal properties (Alzohairy, 2016)
8	Lactapiperanol C		antidiabetic (Daou <i>et al.</i> , 2022)
9	Bruceantin		antineoplastic, antiamebic and antimalarial (National Cancer Institute)

Table 1. Chemical constituents and properties of the phyto-components identified in the aqueous extracts of the leaves of *A. indica* by LC–MS

Name	Formula	RT	Mass	m/z	Positive/ Negative ion mode
8-Hydroxy-2-chlorodibenzofuran	C ₁₂ H ₇ ClO ₂	0.88	218.0129	241.0024	(M+Na) ⁺
(+/-)-3-[(2-methyl-3-furyl)thio]-2-butanone	C ₉ H ₁₂ O ₂ S	0.904	184.0571	185.0643	(M+H) ⁺
Anthranilic acid	C ₇ H ₇ N ₂ O ₂	1.219	137.047	138.0543	(M+H) ⁺
Retronecine	C ₈ H ₁₃ N ₂ O ₂	1.243	155.0941	156.1011	(M+H) ⁺
Adenine	C ₅ H ₅ N ₅	1.274	135.0538	136.0611	(M+H) ⁺
8-Hydroxyadenine	C ₅ H ₅ N ₅ O	1.318	151.0486	152.0559	(M+H) ⁺
2-Methylbenzaldehyde	C ₈ H ₈ O	1.411	120.0567	121.064	(M+H) ⁺
Neuraminic acid	C ₉ H ₁₇ N ₂ O ₈	1.48	267.0937	268.1021	(M+H) ⁺
Benzocaine	C ₉ H ₁₁ N ₂ O ₂	1.804	165.0783	166.0856	(M+H) ⁺
2-Hexylbenzothiazole	C ₁₃ H ₁₇ N ₂ S	2.277	219.1095	220.1168	(M+H) ⁺
N-Acetylserotonin	C ₁₂ H ₁₄ N ₂ O ₂	2.804	218.1048	219.1121	(M+H) ⁺
3-Carboxy-4-methoxy-N-methyl-2-pyridone	C ₈ H ₉ N ₂ O ₄	2.996	183.0542	206.0432	(M+Na) ⁺
Metipranolol	C ₁₇ H ₂₇ N ₂ O ₄	3.036	309.1903	310.1995	(M+H) ⁺
Indoleacrylic acid	C ₁₁ H ₉ N ₂ O ₂	3.093	187.0622	188.0695	(M+H) ⁺
L-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	3.136	204.0887	205.0959	(M+H) ⁺
Isocarbostyryl	C ₉ H ₇ N ₂ O	3.14	145.0519	146.0591	(M+H) ⁺
6-Methylquinoline	C ₁₀ H ₉ N	3.146	143.0726	144.0799	(M+H) ⁺
D-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	3.468	204.0874	205.0945	(M+H) ⁺
Carbofuran	C ₁₂ H ₁₅ N ₂ O ₃	3.602	221.1068	222.114	(M+H) ⁺
Methaphenilene	C ₁₅ H ₂₀ N ₂ S	3.687	260.1351	261.1431	(M+H) ⁺
Venlafaxine	C ₁₇ H ₂₇ N ₂ O ₂	3.969	277.2011	300.1902	(M+Na) ⁺
ibopamine	C ₁₇ H ₂₅ N ₂ O ₄	4.037	307.1766	308.1839	(M+H) ⁺
Dihydrocorynantheine	C ₂₂ H ₂₈ N ₂ O ₃	4.199	368.2095	391.1986	(M+Na) ⁺
Elaeokanine C	C ₁₂ H ₂₁ N ₂ O ₂	4.435	211.1559	212.1632	(M+H) ⁺
Thesinine 4'-O-glucoside	C ₂₃ H ₃₁ N ₂ O ₈	4.555	449.1985	450.206	(M+H) ⁺
2-Heptylfuran	C ₁₁ H ₁₈ O	5.093	166.1367	189.1259	(M+Na) ⁺
Istamycin C1	C ₁₉ H ₃₇ N ₅ O ₆	5.274	431.27	432.2772	(M+H) ⁺
Ascorbyl stearate	C ₂₄ H ₄₂ O ₇	5.365	442.287	443.2946	(M+H) ⁺
Progeldanamycin	C ₂₇ H ₄₁ N ₂ O ₆	5.605	475.2959	476.3033	(M+H) ⁺
(x)-1-Nonen-3-yl acetate	C ₁₁ H ₂₀ O ₂	5.709	184.1471	207.1363	(M+Na) ⁺
Quercetin	C ₁₅ H ₁₀ O ₇	5.864	302.0404	394.7163	(M+H) ⁺
Maritimetin	C ₁₅ H ₁₀ O ₆	6.167	286.0459	287.0532	(M+H) ⁺
Chrysophanol 1-triglucoside	C ₃₃ H ₄₀ O ₁₉	6.172	740.2122	741.2195	(M+H) ⁺
3-Oxo-12,18-ursadien-28-oic acid	C ₃₀ H ₄₄ O ₃	6.447	452.3328	453.3401	(M+H) ⁺
Kuwanon Z	C ₃₄ H ₂₆ O ₁₀	6.521	594.1547	595.162	(M+H) ⁺
6-C-Galactosylluteolin	C ₂₁ H ₂₀ O ₁₁	6.593	448.0974	449.1047	(M+H) ⁺
Thalicssessine	C ₂₂ H ₂₇ N ₂ O ₄	6.727	369.1918	370.1988	(M+H) ⁺
Menthanol	C ₁₀ H ₂₀ O	7.55	156.1525	179.1417	(M+Na) ⁺
Bruceantin	C ₂₈ H ₃₆ O ₁₁	7.759	548.2312	549.2394	(M+H) ⁺
Cucurbitic acid	C ₁₂ H ₂₀ O ₃	7.817	212.1392	213.1473	(M+H) ⁺
6-Methyl-2-methylene-6-octene-1,3,8-triol	C ₁₀ H ₁₈ O ₃	8.083	186.126	209.1153	(M+Na) ⁺
6,6-Dimethoxy-2,5,5-trimethyl-2-hexene	C ₁₁ H ₂₂ O ₂	8.329	186.1631	209.1523	(M+Na) ⁺
2-(3-Phenylpropyl)tetrahydrofuran	C ₁₃ H ₁₈ O	8.665	190.1345	191.142	(M+H) ⁺
(1R,2R)-1,2,7,7-Tetramethylbicyclo[2.2.1]heptan-2-ol	C ₁₁ H ₂₀ O	8.93	168.1523	191.1417	(M+Na) ⁺
Geranyl 2-ethylbutyrate	C ₁₆ H ₂₈ O ₂	9.949	252.2096	275.1989	(M+Na) ⁺
10,16-dihydroxy-palmitic acid	C ₁₆ H ₃₂ O ₄	10.863	288.2301	311.2194	(M+Na) ⁺

2-Tetradecylcyclobutanone	C18H34O	11.116	266.2617	289.251	(M+Na)+
Lactapiperanol C	C16H26O4	11.663	282.1813	283.1889	(M+H)+
L-Menthyl (R,S)-3- hydroxybutyrate	C14H26O3	11.725	242.1887	265.1779	(M+Na)+
Aurachin D	C25H33NO	12.176	363.2605	386.2517	(M+Na)+
5-Methylthioribose	C6H12O4S	4.813	180.0441	179.0367	(M-H)-
Gentisic acid	C7H6O4	2.014	154.0281	153.0209	(M-H)-
2-Mercapto-3-pentanone	C5H10OS	4.09	118.0437	163.0417	(M-H)-
L-Lyxose	C5H10O5	1.145	150.0547	149.0475	(M-H)-
Caulerpin	C24H18N2O4	5.686	398.1267	443.1249	(M+HCOO)-
Copalliferol B	C42H32O9	6.148	680.2045	739.2189	(M+HCOO)-
Allivicin	C27H30O16	6.149	610.1599	609.1526	(M-H)-
Aprepitant	C23H21F7N4O3	6.532	534.1448	593.1586	(M+CH3COO)-
CMP-N-glycolylneuramate	C20H31N4O17P	6.568	630.1409	629.1353	(M-H)-
Dopaxanthin quinone	C18H16N2O8	7.099	388.086	447.0999	(M+CH3COO)-
Cynaroside	C21H20O11	7.838	448.1062	447.0989	(M-H)-
Physalin O	C28H32O10	8.784	528.1937	527.1868	(M-H)-
Corchorifatty acid F	C18H32O5	9.899	328.2302	327.2227	(M-H)-
Oxazepam	C15H11ClN2O2	9.931	286.0523	285.0449	(M-H)-
N-Desmethyloclobazam	C15H11ClN2O2	10.312	286.0522	285.0448	(M-H)-
2R-hydroxy-stearic acid	C18H36O3	11.14	300.2708	359.2846	(M+CH3COO)-
Chloramphenicol succinate	C15H16Cl2N2O8	12.487	422.0334	481.047	(M+CH3COO)-
Lauryl hydrogen sulfate	C12H26O4S	18.992	266.1583	265.151	(M-H)

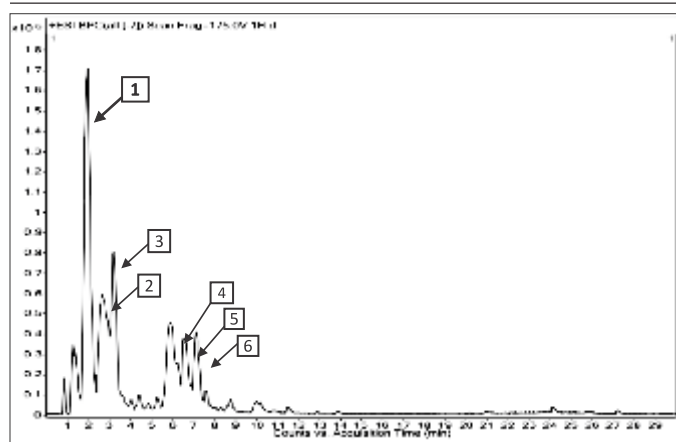


Fig.1(a). HR-LC/MS spectrum peak of *A. indica* aqueous extract showing the chromatogram intensity against the acquisition time, (A) positive ion mode analysis. Numerical 1- Benzocaine; 2-Metipranolol; 3-Indoleacrylic acid; 4-Quercetin; 5- Maritimetin and 6-Menthanol

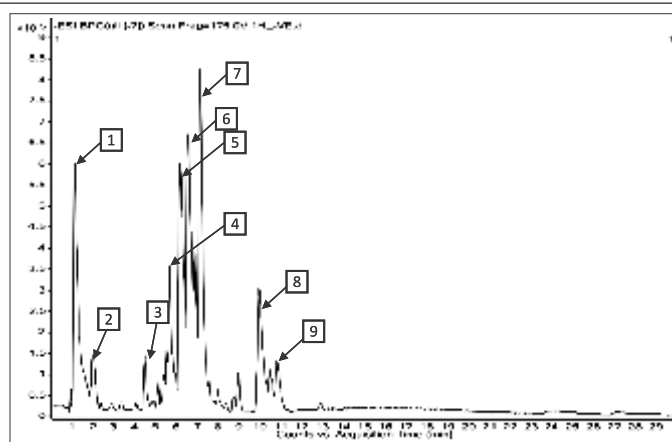


Fig.1(b). HR-LC/MS spectrum peak of *A. indica* aqueous extract showing the chromatogram intensity against the acquisition time, negative ion mode analysis. Numerical 1-L-Lyxose; 2-Gentisic acid; 3-5-Methylthioribose; 4-Caulerpin; 5-Allivicin; 6-Copalliferol B; 7-Cynaroside; 8-N-Desmethyloclobazam; 9-Corchorifatty acid

by interacting with free radicals and reducing oxidative damage (Nita and Grzybowski, 2016). These compounds stabilize or deactivate free radicals, frequently before they attack targets in biological cells and have reportedly been found in medicinal plants (Rahmani *et al.*, 2015). Flavonoids have been present in plants for more than a billion years and have a wide range of biological functions, but antioxidants are one of their most important biological functions (Anand David *et al.*, 2016). According to Prashanth *et al.* (2015), these polyphenolic chemicals are present in neem leaf extracts that are both aqueous and ethanolic. The most notable characteristic of quercetin is

its antioxidant activity. It seems to be the most powerful flavonoid for protecting the body against reactive oxygen species, produced during the normal oxygen. The reactivity of the extracts prepared from *A. indica* was analyzed with 2, 2-Diphenyl-1-Picryl hydrazyl, a stable free radical that reduced by accepting hydrogen or electron from the donor molecule. The extent of DPPH radical scavenging (Fig. 3) at different concentrations (0.2-1.0 mg/ml) of *A. indica* extract was measured with ascorbic acid as the standard. In comparison with ascorbic acid, the plant extract had a lower level of inhibitory activity. The

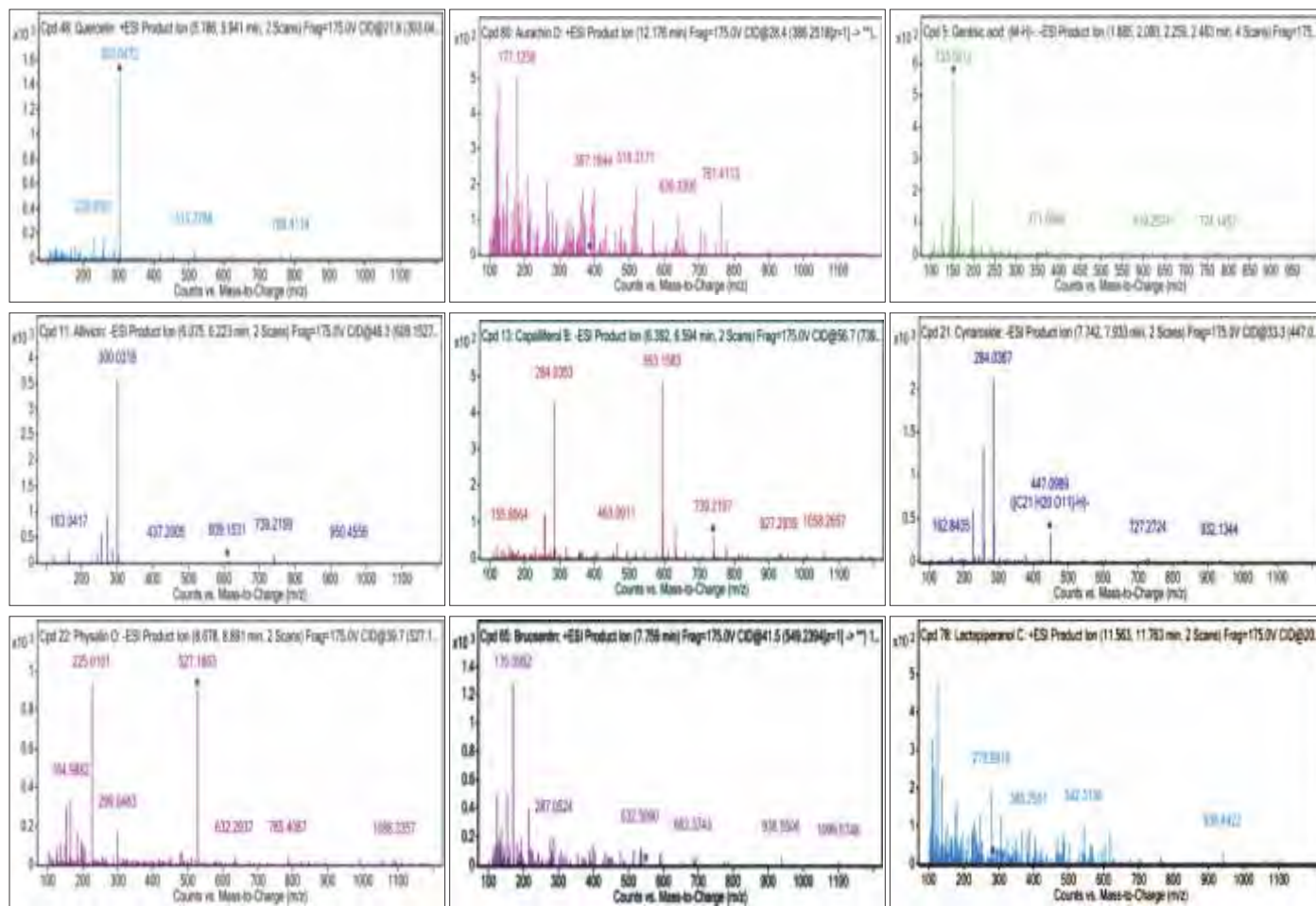


Fig. 2. Typical direct flow injection analysis ESI-MS fingerprint spectra of above discussed phytochemicals from the leaves of *A. indica*

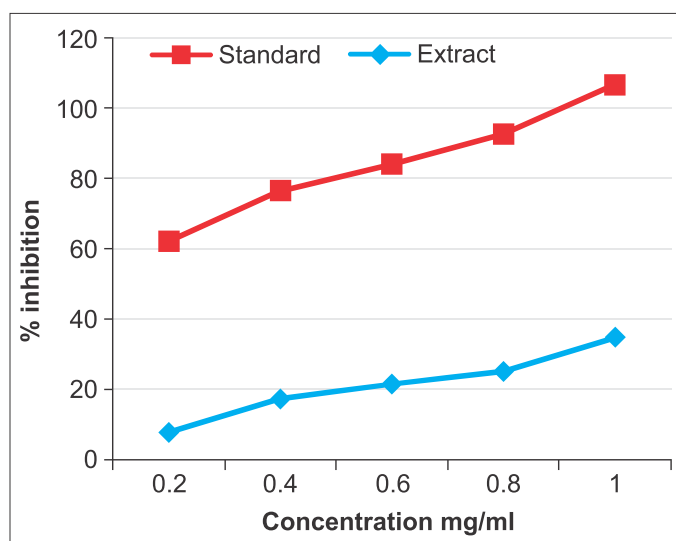


Fig. 3. DPPH radical scavenging activity of different concentrations of aqueous leaf extract, of *A. indica* and ascorbic acid (standard)

results showed that the greatest degree of inhibition activity was seen at higher concentrations of plant extract, followed by decreasing inhibition activity at lower concentrations. Neem tree leaf extracts had free radical scavenging properties that were found to be effective against the DPPH radical.

CONCLUSION

The present chromatographic study provides abundant information for structural elucidation of the chemical compounds present in *A. indica* aqueous leaf extract especially when tandem mass spectrometry (MS) is applied. Consequently, the combination of HPLC and MS provides a quick and precise method for phytochemical identification and points out that extracts of *A. indica* leaves comprise a variety of compounds in appreciable quantity that have preventive and therapeutic efficacy against different pathological conditions and protect the body against the harmful effects of oxidative stress caused by free radicals, therefore, they can be used as a source of potent natural antioxidant.

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