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Research Article

Characterization of Curculigo Orchioides And Asparagus Racemosus

Identification And Characterization Of Phenolic Compounds In Root Extract Of Two Ethnomedicinal Plants *Curculigo Orchioides* And *Asparagus Racemosus*

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Abstract: Since early ancient period human being are searching for new drugs with better therapeutic potentials. Traditional medicines are very important because it provide the right direction to the researcher to discover new plant based products against some specific problem and the knowledge has been passed through generation after generation. *Curculigo orchioides* Gaertn.and *Asparagus racemosus* Willd. both plants have immune stimulatory properties and are widely used by traditional healers for the treatment of various diseases. The main aim of the present study is to identify the secondary metabolites found in methanolic root extracts of *C. orchioides* and *A. racemosus*. Extraction was carried out through standard procedures and the analysis of plant extracts was carried out by using LC-ESI-MS/MS technique in positive and/or negative ionization mode. LC MS study tentatively identified 15 and 19 secondary metabolites from *Curculigo orchioides* and *Asparagus racemosus* respectively. Amongst these some were simple phenolic acids such as caffeic acid, quinic acid, p-coumaric acid, sinapic acid, protocatechuic acid, p-hydroxybenzoic acid and vanillic acid. Some others were phenolic acid esters such as chlorogenic acid, di caffeoylquinic acid, p-coumaroyl quinic acid and some flavonoids such as quercetin, rutin, kaempferol, catechin and apigenin were also tentatively identified. Two phenolics caffeic acid and caffeoyl hexoside were further confirmed by MS MS study. This study supports the ethnobotanical claims done by traditional healers of Purulia and Midnapore districts. Characterized phytochemicals were mainly phenolic and flavonoid compounds. Assured levels of phenolics along with other plant constituents in the studied plants supports the ethnobotanical claim done by traditional healers. Further research is needed on structural analysis and bioactivity assay of the identified compounds.

Keywords: *Asparagus racemosus, Curculigo orchioides*, LC-ESI-MS/MS analysis, Mass fragmentation, Secondary metabolites.

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1. INTRODUCTION

India is known as a large storehouse of medicinal herbs from which raw materials are collected by indigenous people, mainly by traditional healers for the treatment of different diseases.[1] Due to low cost, fewer side effects and availability, herbal medicines have attracted attention to cure health problems by rural indigenous human beings.[2] The knowledge about traditional treatment is usually provided by folk medicine practitioners. They collect plant materials to prepare the orthodox medicine either as a raw material or in dry condition with or without necessary ingredients to maintain their correct dosage.[3]This knowledge is transmitted from generation to generation through their conversation. However, at present their knowledge of ethnomedicine is slowly declining due to their modern lifestyle and lack of interest. The only way to preserve ancient knowledge is to collect, store and document the information collected from the tribal people.[4] Scientific studies of ancient researchers suggested that herbal medicines could be a way to cure human illness because plants generally consist of many active bio-compounds.[2,5] These organic compounds are primarily responsible for curing several health problems such as fever, diarrhea, jaundice, common cold, dysentery, skin diseases, abdominal pain, inflammation, conjunctivitis etc. Some selective plants also play an important role in fatal health problem such as diabetes, gastrointestinal problems, heart disease, abnormalities of kidney function, cancer etc.[6-12] Medicinal plants *Curculigo orchioides* and *Asparagus racemosus*, commonly known as Kali musli and Shatamuli have the property to cure several common to complicated problematic health issues. They are often used as domestic medicine in our country. *C. orchioides* Gaertn. belongs to the Hypoxidaceae family which is a perennial herb.[13] Root is oblong and black brown in colour externally. It bears bright yellow-coloured flowers and leaves are simple, lanceolate and crowded with a sheathing base. Rhizomes of this plant are widely used as medicine in our country.[14, 15]It has been used to cure vomiting, kidney problems , leucorrhea, skin disease, asthma, bronchitis, jaundice, cancer, piles, leukoderma and also as aphrodisiac, antioxidant, antidiabetic, immunoadjuvant, antibacterial and neuroprotective.[16, 17]The reported active bio-compounds are flavonoids, phenolic compounds, glycosides, alkaloids, saponins, and other secondary metabolites.[15, 18]*C. orchioides* have been reported to contain many polyphenolic compounds such as curculigoside, a colourless, needle shaped polyphenol.[18] It is also reported that flavone glycoside from the root has been identified as 5, 7-dimethoxy glucopyranoside and also various types of fatty acids like palmitic acid, oleic acid, linoleic acid, arachidic acid have been isolated from root oil.[19] Active phyto-components identified from the extract of rhizome by GC-MS are ethyl iso-allocholate (steroid compound), docosanoic acid 1, 2, 3-propanetriyl ester (Tribehenin) as a fatty acid ester, benzoic acid, 4-ethoxy, ethyl ester (aromatic acid ester).[13]*A. racemosus* Willd., a universally accepted medicinal plant belongs to the family Asparagaceae and is commonly known as Shatamuli or Shatavari.[20] It is a woody climbing plant consisting of needle like leaves with small white flowers.[21]The natural medicine obtained from *A. racemosus*is widely used by indigenous people of India. Literature review showed that about 25% of modern medicine available for the treatment of diseases originates from natural products.[12] It is commonly used to cure epilepsy, kidney disorder, chronic fevers, excessive heat, stomach ulcers, nervous disorder, dyspepsia, tumors, neuropathy, inflammation, liver cancer and also increase milk secretion in nursing mother.[22] It also possesses major biological activities as antioxidant, antidepressant, anti-hepatotoxic, immunomodulant, hemolytic, antibacterial, hypocholesterolemic nematicide,5-alpha-reductase inhibitor, anti-cancer and have activity in neuronal damage.[22, 23] It has also been useful for the treatment of diabetes.[24] Review on phytochemical studies reveals that it contains active constituents like saponins (Shatvarin IX, IV, V, Asparanin A), immunoside, asparagamine A, racemofuran, hyperside, polycyclic alkaloid and also other primary chemical constituents are essential oils, camphor, decanoic acid, flavonoids of kaempferol, quercetin, and rutin.[20, 25]Local tribal healer "Kabiraj" learned the formulation of drugs from his ancestral lineage which is passing through generation to generation. Though earlier research dealing with different aspects of conventional ethnobotanicals from different parts of the state has been carried out, only a few of them have been devoted to the secondary metabolite analysis. On that account, the main objective of this research work was to characterize the fraction obtained from methanolic extract of *A. racemosus* and *C. orchioides* by using high performance liquid chromatography-diode array detection (HPLC-DAD) integrated with ESI-mass spectrometry (ESI-MS). Here, the analyzed MS data with interpreted ion fragments was confirmed by comparison with the fragmentation pattern and MS spectra of reference literature data. It is believed that this work can provide comprehensive information for quality assessment.

2. MATERIALS AND METHODS

2.1 Plant material preparation

Asparagus racemosus[Figure 1] was collected from the Godapiasal forest (Latitude: 22.5642° N and Longitude: 87.2792° E) of Paschim Medinipur district and *Curculigo orchioides* [Figure 2] was collected from Bandwan forest (22°52'33.6"N; 86°30'25.2"E) of Purulia district in West Bengal, India. Both the plants were identified by Dr. Shyam Biswas, Botanical Assistant at the Central National Herbarium, Botanical Survey India, Kolkata. Voucher specimen number for *C. orchioides* and *A racemosus* is GM-06 and VU-01 respectively. Collected roots were washed with running tap water to remove soil and dirt. Then these roots were dried under shade during sunny days for approximately 25-30 days and powdered by using a grinding machine.

Fig 1: Sampling of *Asparagus racemosus*.

Fig 2: *Curculigo orchioides* with underground parts.

2.2 Extraction Procedure

Root extracts of *Asparagus racemosus* and *Curculig oorchioides* were treated with 2:1:1 methanol, chloroform and water and kept for 72 hrs. at 4°C.The methanolic water phase was taken for further analysis while the chloroform phase was discarded to eliminate the lipid present. Chilled acetone was added to the aqueous methanolic phase in 4:1 (v/v) ratio. Solution was incubated at -20°c for 1 hour and then centrifuged at 12,000 rpm at 4°c for 15 min. Protein precipitate was rejected and the supernatant was collected, evaporated by rotary evaporator and subjected for analyzing LC-MS/MS. [26]

2.3 Chromatographic conditions

Samples were separated onto reversed phase-HPLC (Agillent 1100 series, USA) by using ZORBAX300-SB C18 column (4.6 mm × 150 mm, particle size 5 µm) operated at 30°C.[27] Gradient of two mobile phases were used: methanol (A) and water with 0.02% aqueous H_3PO_4 (B), set at 25% A+ 75% B for 5 min> 30% A+ 70% B for 10 min> 45% A + 55% for 30 min> and 80% A + 20% B for 15 min. The injection volume was 10 µl. The flow rate was kept at 1mL min^{-1} and the analytes scanned from 220 nm to 520 nm and chromatograms were monitored at 280 nm wavelength.

2.4 LC-MS/MS Analysis

Purified HPLC fraction analysis were carried out by using LC-MS/MS system (Water, USA), interfaced with a Micromass Quattro micro triple quadrupole mass spectrometer (Micromass, Manchester, UK) and Masslynx software.[28] Sample was introduced carefully by using sample loaded syringe pump at a flow rate of 10µl min^{-1} and injection volume was 100µL. Mass spectrometer was operated in negative and positive electrospray ionization mode and mass spectra of the column elutes were recorded by scanning the mass range from m/z 50 to 1500 high purity , N_2 gas at a flow rate 10.0 ml/min was used as nebulising gas with collision energy of 30ev. The heated electrospray source temperature and desolvation temperature of 130°C, 300°C were used, maintaining the scan time of 0.5sand inter scan delay time of 0.1s.[28]

3. RESULTS

In the present work, a qualitative analysis of the secondary metabolites from methanol extract of *C. orchioides* and *A. racemosus* has been carried out using HPLC-ESI-MS in negative and positive ionization modes.

3.1 Analysis of secondary metabolites in *C. orchioides*

Figure 3, A-C corresponds to the base peak chromatogram (BPC) in positive and negative ionization modes together with the UV chromatogram at 280 nm in methanol extract. Fifteen compounds tentatively identified through HPLC-ESI-MS experiments along with their retention time (t_R), m/z values either positive and / or negative ionization mode, the names of the compounds were shown in Table 1. In *C. orchioides* MS profiling of peak 1 with a $[M-H]^-$ ion at m/z 367 yielding a quinic acid ion at m/z 191 by loss of a ferulic acid moiety was tentatively identified as feruloyl-quinic acid and another fragment ion at m/z 229 suggesting the addition of chlorine with quinic acid. Likewise, Peak 2 and 3 at m/z 153 and 175 were tentatively identified as protocatechuic acid and ascorbic acid respectively. Peak 4 at m/z 179 indicates caffeic acid moiety and another peak at m/z 152 indicates loss of water. Peak 8 at m/z 167 identified as vanillic acid and another peak at m/z 215 indicates the addition of chlorine. Molecular ion peak 9 at m/z 137 was probably identified as p–hydroxy benzoic acid. Likewise, in the negative mode at retention time 16.410 characterizes the presence of quercetin hexose malic acid. The fragment ion obtained at m/z 464 suggesting the loss of quercetin hexose moiety. Peak 7 at m/z 355 was tentatively identified as caffeoylquinic acid. Fragment ion at m/z 181 suggesting the loss of caffeic acid moiety. Similarly, other peaks present in the figure were tentatively identified as shown in Table 1

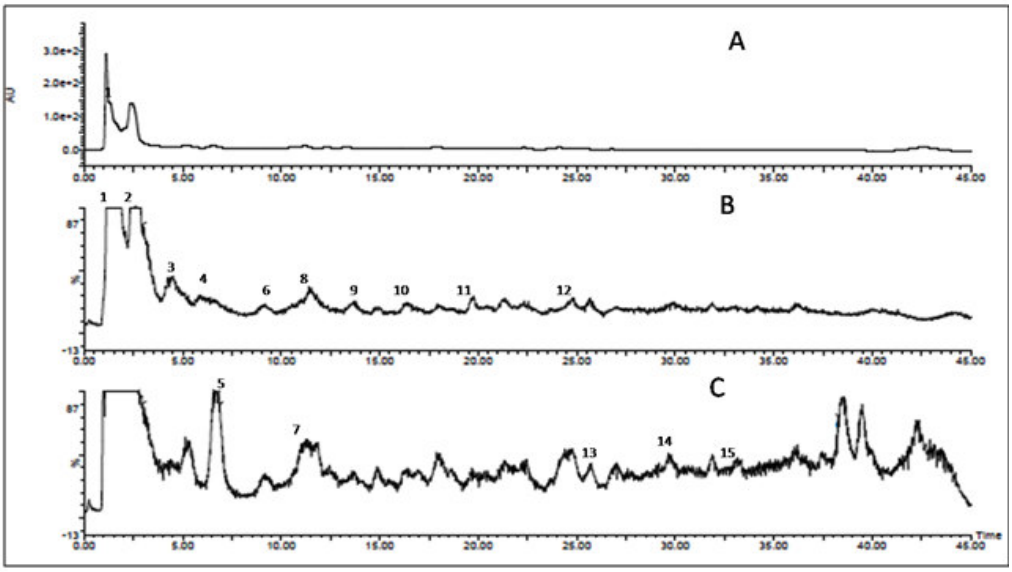

Fig 3: UV chromatogram obtained at 280 nm (A), LC-ESI-MS base peak chromatogram of *C. orchioides* in negative (B) and positive (C) ion mode. (X = Retationtime; Y axis =Relative abundance; Peak no. mentioned in the figure are respective compounds as noted in table 1).

Table 1: Tentative identification of secondary metabolites obtained through LC-ESI-MS positive and negative mode in *C. orchioides*.

Peak no.	RT	$[M-H]^-$ m/z	$[M+H]^+$ m/z	Name of the compound	MW	Reference
1.	1.348	191		Quinic acid	192	Sana Bakari et al, 2018[51]
2.	2.512	153		Protocatechuic acid	154	Ibrahim M. Abu-Reidah et al, 2015[48]
3.	4.502	175		Ascorbic acid	176	May A. El-Sayed et al, 2017[58]
4.	5.919	179		Caffeic acid	180	Sunil Kumar et al, 2017[49]
5.	6.703		165	p-Coumaric acid	164	Ibrahim M. Abu-Reidah et al, 2015[48]
6.	9.182	359	361	Caffeic acid dimer		Ibrahim M. Abu-Reidah et al, 2015[48]
7.	11.291		355	Caffeoylquinic acid		Ibrahim M. Abu-Reidah et al, 2013[59]
8.	11.400	167		Vanillic acid	168	May A. El-Sayed et al, 2017[58]
9.	13.728	137		p-hydroxy benzoic acid	138	Ibrahim M. Abu-Reidah et al, 2015[48]
10.	16.410	579		Quercetin hexose malic acid	312	HaticeTohma et al, 2016[57]
11.	19.766	311		Caftaric acid		Ibrahim M. Abu-Reidah et al, 2015[48]
12.	24.776	515		Dicaffeoylquinic acid		Ibrahim M. Abu-Reidah et al, 2013[59]
13.	25.687	609		Rutin	610	Sana Bakari et al, 2018[51]
14.	29.937	301	303	Quercetin	302	Yeqing Chen et al, 2015[54]
15.	33.167		377	Caffeic acid derivative		Fabiana Della Betta et al, 2018[56]

3.2 Analysis of secondary metabolites in *A. racemosus*

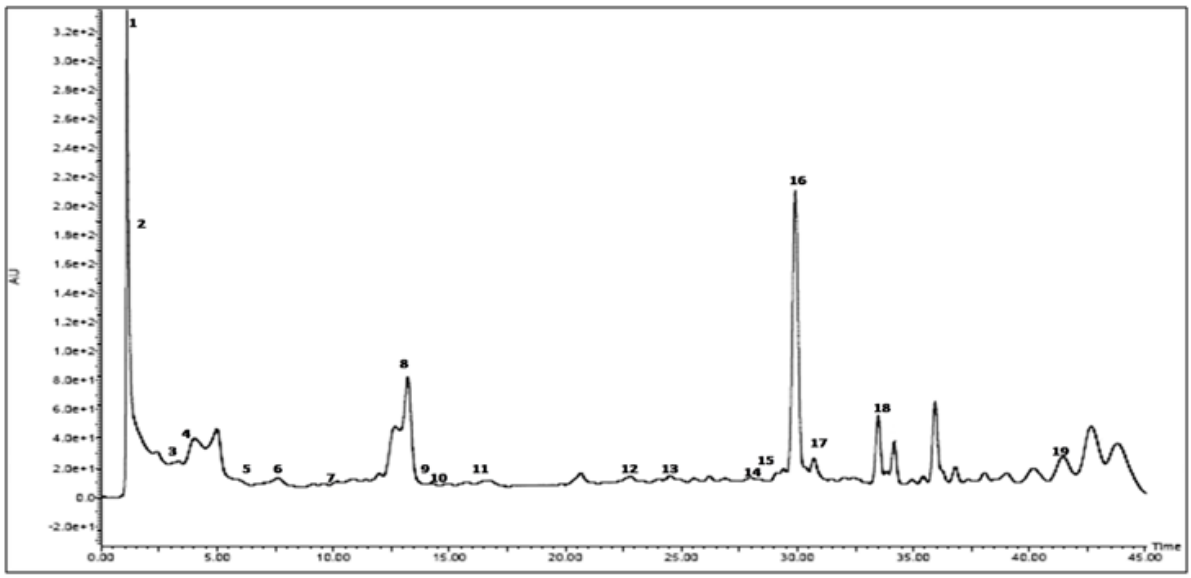

Fig 4: HPLC chromatogram of *A. racemosus*. (X axis= Retention time; Y axis =Relative abundance; Peak no. mentioned in the figure are respective compounds as noted in Table 2).

Nineteen peaks [Figure 4] were tentatively identified from *A. racemosus* as shown in Table 2. Peak 1 at m/z 540 in negative ionization mode and 542 in positive ionization modes was tentatively identified as caffeic acid derivative due to the presence of molecular ion peak at m/z 179 and 181, which is the characteristic molecular peak of caffeic acid in negative and positive modes respectively. Peak 2 was identified as caffeic acid as MS^2 study of this peak identified two fragment peaks [Figure 5] one is m/z 161 ($M-H_2O$) due to loss of water and another one at m/z 135 ($M-CO_2$) due to loss of carbon dioxide which are the characteristic fragment peaks of caffeic acid. Peak 3 with m/z 353 was identified as chlorogenic acid due to the presence of molecular ion peak at m/z 179 and 191.Peak 4 was identified as caffeoyl -o-hexoside [Figure 6] as MS^2 study revealed the presence of two fragment peaks at m/z 179 and 161. Peak 5 with m/z 223 was tentatively identified as sinapic acid; molecular peak at m/z 385 indicates addition of hexose moiety and another peak at m/z 447 suggesting (2M-1). Peak 6 at m/z 285 was tentatively identified as kaempferol. Peak 7 was tentatively identified as catechin due to the presence of molecular peak at m/z 579 indicates (2M-1) and another peak at m/z 145 due to loss of (-o-CH_2 CH_3). Peak 8 at m/z 447 tentatively identified as luteolin hexoside and another peak at m/z 285 suggesting the loss of hexose. Peak 8 at m/z 449 in positive ionization mode also confirmed the compound luteolin hexoside. Peak 9 at m/z 269 identified as apigenin and another peak at 315 suggesting the addition of $HCOO^-$, likewise another peak at m/z 540 suggesting (2M-1). Peak 10 was identified as di-caffeoylquinic acid due to the presence of peak at m/z (2M-1) and another peak at m/z 181. Peak 11 was tentatively identified as quinic acid due to the presence of molecular peak at m/z 191 and 193 in positive and negative ionization mode respectively. Likewise other peaks were identified on the basis of $(M+H)^+$ and/ or $(M-H)^-$ value as shown in Table 2.

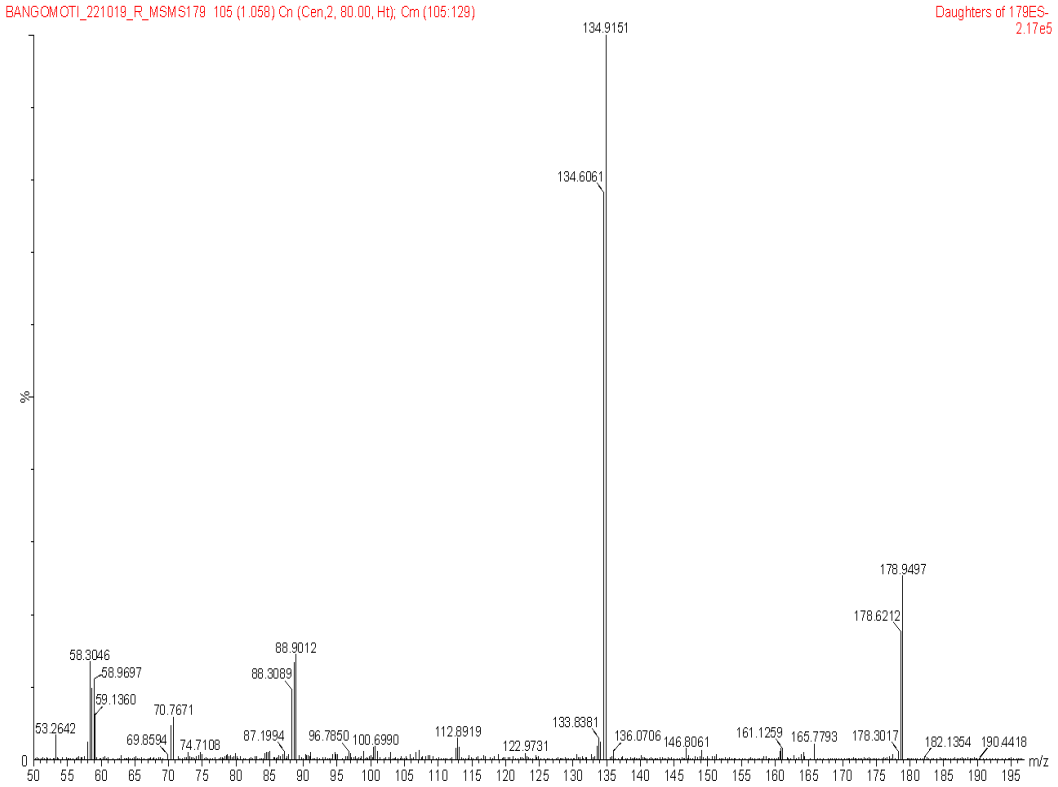

Fig 5: Mass fragmentation of m/z 179; X axis= Relative m/z value; Y axis= Relative abundance

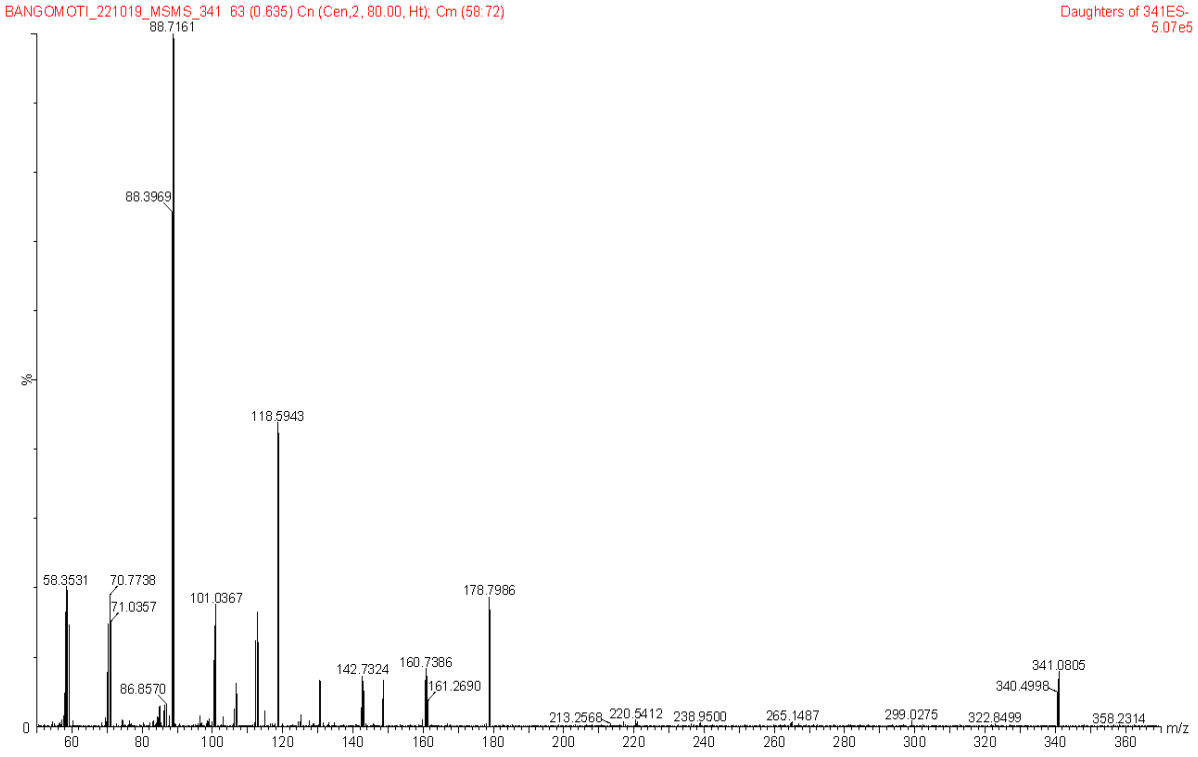

Fig 6: Mass fragmentation of m/z 341; X axis= Relative m/z value; Y axis= Relative abundance

Table 2: Tentative identification of secondary metabolites obtained through LC-ESI-MS positive and negative mode in *A. racemosus.*

Peak No	Rt(min)	$[M-H]^-$ (m/z)	$[M+H]^+$ (m/z)	Name of the compound	MW	Reference
1	1.308	540	542	Caffeic acid derivative		May A. El-Sayed et al, 2017[58]
2	1.350	179	-	Caffeic acid	180	Sunil Kumar et al, 2017[49]
3	2.785	353	-	Chlorogenic acid	354	S. Demiray et al, 2009[52]
4	3.662	341	-	Caffeoyl-O- hexoside	342	Ridha Ben Said et al, 2017[53]
5	5.991	223	-	Sinapic acid	224	J. Oszmianski et al, 2013[47]
6	7.763	285	-	Kaempferol	286	Ibrahim M. Abu-Reidah et al, 2015[48]
7	10.278	289	-	Catechin	290	ImenBelhadjSlimen et al, 2017[50]
8	13.080	447	449	Luteolinhexoside		Ibrahim M. Abu-Reidah et al, 2015[48]
9	14.447	269	-	Apigenin	270	ImenBelhadjSlimen et al, 2017[50]
10	14.506		517	Dicaffeoylquinic acid		Ibrahim M. Abu-Reidah et al, 2015[48]
11	16.928	191	193	Quinic acid	192	Sana Bakari et al, 2018[51]
12	23.442	283	-	Acacetin	284	Sana Bakari et al, 2018[51]
13	24.725	343		Dihydro-caffeoyl-O-glucoside		Ibrahim M. Abu-Reidah et al, 2013[59]
14	28.193		339	P-Coumaroylquinic acid	338	May A. El-Sayed et al, 2017[58]
15	29.180	609	-	Rutin	610	Sana Bakari et al, 2018[51]
16	30.024	383		1,3-O- Dicoumaroylglycerol		May A. El-Sayed et al, 2017[58]
17	30.783	443		1,3-O-Diferuloylglycerol		HaticeTohma et al, 2016[57]
18	33.636	331		Galloylhexose		Azila Abdul Karim et al, 2014[55]
19	41.599		315	Caffeic acid derivative		May A. El-Sayed, 2017[58]

4. DISCUSSION

Continuous production of reactive oxygen species leads to the emergence of oxidative stress in the body which can be controlled by administering antioxidants. Most of the antioxidants belong to the family of phenolic compounds.[29] On the basis of mass fragmentation study authors tentatively identified some phenolic acids, phenolic acid esters and flavonoids. Amongst them caffeoylquinic acid; caffeic acid and quinic acid all have antioxidant activity. Results obtained in this study supports the previous report of Chen (2016). In addition, caffeoylquinic acid also shows antiviral activity and DNA protective activity.[30] LC MS study tentatively identified 15 and 19 secondary metabolites from *Curculigo orchioides* and *Asparagus racemosus* respectively. Amongst these some were simple phenolic acids such as caffeic acid, quinic acid, p-coumaric acid, sinapic acid, protocatechuic acid, p-hydroxybenzoic acid and vanillic acid, some phenolic acid esters such as chlorogenic acid, Di caffeoylquinic acid, p-Coumaroyl quinic acid and some flavonoids such as quercetin, rutin, kaempferol, catechin and apigenin were identified. Caffeic acid and its derivatives exist as natural phenolic compounds with a wide range of biological activities such as prevention of cancer cell proliferation, antioxidant activity, anti-aging and anti-inflammatory activity as well as anti-diabetic activity.[31]Quinic acid demonstrates antiviral activities against HIV, Hepatitis B Virus and Herpes Simplex Virus 1. Further studies have revealed also that quinic acid has anti-dengue activities.[32]Sinapic acid has the ability to inhibit dangerous radicals to reduce oxidative stress and also tested against inflammation, cancer, diabetes, neurodegeneration and anxiety.[29]Protocatechuic acid has similar structure to that of caffeic acid which is well known for its antioxidant activity. Protocatechuic acid have different biological activities such as antibacterial, anticancer, antiulcer, antidiabetic, antiviral, cardiac, antiatherosclerotic, hepatoprotective, neurological and nephroprotective activity.[33]Catechin is a type of biological compound that provides various health advantages like uv-protection activity, antimicrobial activity, antiviral and anti-cancer activity. It also has a significant role in the inhibition of the production of matrix metalloproteinase enzymes.[34]Research evidence claims that chlorogenic acid can regulate glucose and lipid metabolism *invivo* in both cases of healthy and genetically modified metabolic disorder condition.[35]Caftaric acid is a polyphenol oxidase has hyaluronidase inhibitory activity, antioxidant effect and increase of insulin secretion.[36]Vanilin is an oxidized form of vanillin has biological activity against chronic intestinal inflammation. It has been studied that Vanillic acid affects Dextrane Sulfate Sodium (DSS) induced ulcerative colitis.[37]Ascorbic acid, known to us as Vitamin C, is of great interest to researchers in the prevention of cataracts, which is the leading cause of blindness in the world.[38]Flavonoids are a type of polyphenolic compounds that belong to secondary metabolites of plants.[39]There are various medicinal aspects of flavonoids in human health along with biological activities.[40]Out of 6000 different flavonoids, Quercetin, Kaempferol, Apigenin, Luteolin, Rutin, Acacetin are the most ever-present flavonoids.[39]Quercetin is important for its anti-inflammatory, vasodilator effects, antiobesity, antihypercholesterolemic, antihypertensive and anti aetherosclerotic acitivities.[41]Many studies have gained interest in flavonoid Kaempferol because of its ability to reduce the risk of chronic disease. Kaempferol can modulate cellular signaling system linked to apoptosis, angiogenesis, inflammation and metastasis. It can reduce cancer cell proliferation and help to prevent cancer cell death.[40] Researchers have also shown that flavonoid Apigenin plays an important role in the treatment of cancer cells including cancer chemoprevention and drug interactions.[39]Chemically Rutin is glucoside which contains flavonoid aglyconequercetin and disaccharide rutinose. It is documented that it has biological activities including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activity.[42]The flavonoid Acacetin has many beneficial activities including antimutagenic, antiplasmodial, antiperoxidase, anti-inflammatory and anti-cancer activity. It helps to prevent cancer cells from spreading through cell death or by blocking the cell cycle.[43] The outcome of this study strongly supports the ethnobotanical claims made by

the traditional healers of Purulia and Midnapore districts. The antibacterial effects and the antioxidant property of *C. orchioides* and *A. racemosus* are due to the presence of the above-mentioned bioactive compounds. Further research is needed to throw light on the structural analysis and bioactivity of the identified bioactive compounds. We can summarize that the synergistic effect of all these chemical/bioactive compounds adds a therapeutic property to the studied plants used by the ethnic community for the treatment of various ailments from time immemorial.[44-46]

5. CONCLUSION

The root extract analysis shows the presence of large numbered phenolic compounds including flavonoids. In this work, qualitatively around 34 active phenolic and flavonoid compounds were separated and tentatively identified from *A. racemosus* and *C. orchioides* by using Liquid Chromatography combined with improved mass spectrometry (LC-MS/MS) technique. This technique can be used for routine analysis of biologically active phytochemicals. The study of previous references also helped to evaluate the therapeutic aspects of these biologically active compounds in human health. Therefore, it can be concluded that the synergistic effect of all these chemical compounds adds a therapeutic property to the studied plants which is used by the ethnic community of Purulia and Midnapore region from time immemorial for curing various ailments. The prospect of this work may be helpful for the discovery of drugs, characterization of natural products and identification of impurities or degradation of plant products in the pharmaceutical study.

6. AUTHORS CONTRIBUTION STATEMENT

Bangamoti Hansda is the main author and wrote this research paper. Ghanashyam Mahato contributed and discussed methodology and results of the manuscript. Arnab Bera contributed in writing references of the final manuscript. Nilanjana Banerjee is the main supervisor of this paper.

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9. CONFLICT OF INTEREST

There is no conflict of interests on behalf of all authors.

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