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HPLC-MS/MS Based Profiling of Antimutagenic Agents of *Digera muriacata* Leaf

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The current study was aimed to characterize antimutagenic agents of *Digera muriacata* by high performance liquid chromatography coupled with mass spectrometry. Extraction procedure was optimized for better recovery of antimutagenic agents using acidified methanol (0.5, 1.0 and 2.0 N) and aqueous methanol (70 %, 50 % and 30 % v/v) to get hydrolyzed and non-hydrolyzed extracts, respectively. Both types of extracts were found to be non-mutagens with hydrolyzed extracts depicting higher antimutagenicity (70.63 ± 0.69 % for TA98 and 73.98 ± 0.70 % for TA100) than those of non-hydrolyzed extracts (68.25 ± 0.69 % for TA98 and 68.29 ± 1.22 % for TA100). Hence, optimized hydrolyzed extract was further characterized for its bioactives by LC-MS/MS analysis which showed the presence of ten compounds including kaempferol-O-dirhamnoside, 5-O-feruloylquinic acid, malvidine-3-O-glucoside, *etc*. These results potentially recommended vast applications of *Digera muriacata* in medicinal formulations for curing various diseases.

Keywords: *Digera muriacata,* LCMS/MS, Antimutagenic activity.

INTRODUCTION

Oxygen containing free radicals and non-radical species are very much reactive agents that can easily interact with various biomolecules like proteins, lipids, carbohydrates and DNA. Such interactions may lead to various degenerative processes in living cells that finally result in different fatal diseases and aging. Moreover, ROS are also leading factors of heart diseases as well as other chronic inflammations which ultimately lead towards different types of cancers [1]. The oxygen containing free radicals include hydroxyl ($OH^{\bullet}$) superoxide anion ($^{\bullet}O_2^-$), peroxyl ($ROO^{\bullet}$), hydroperoxyl ($HOO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals and non-radicals species like hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are all highly reactive species that may interact with human genome resulting in different types of mutational events. Such mutations are harmful to living organisms, leading to many degenerative diseases [2].

With the advancement of information technology, chemophobia is prevailing day by day. People are getting conscious about side effects of synthetic medicines and drugs. They have trust only on natural and plant based medicines. Thus the researches on medicinal plants have been increased throughout the world. Many of the medicinal plants that had been used by traditional physicians in their medicinal formulations, are being explored for their active ingredients. A large number of medicinal plants have been identified and studied for their protective and biological activities in the recent past. Much attention has been diverted to the extraction of these biologically active phytochemicals, their characterization and mode of action [3].

Many of these phytochemicals are present as secondary metabolites in various species of medicinal plants and responsible for therapeutic values of these plants [4]. Plants and plant based natural products have been used for many years as medicine by traditional physicians to treat many fatal diseases and inflammations. A large number of such medicinal plants have been proved to be an outstanding source of bioactive compounds with potent curative properties [2,5]. Modern research revealed the antioxidant potential of various phytochemicals like phenolics extracted from different plant materials. Various herbs and spices containing high concentrations of such phenolic compounds have been extensively used in medicinal formulations to cure several chronic diseases [6].

Extraction is an important process to discharge medicinally active portion from plant tissues which may be in liquid, semi-liquid or in powder form. It is carried out using different solvents and different extraction techniques. Polarities of extraction solvents and prevailing conditions have great impact on yield and bioactivities of these extracts. Optimization of solvent polarity and prevailing conditions is very crucial step to get maximum extract yield and bioactivities. Many

phytochemicals of medicinal values may be bound to various biomolecules in plant intracellular organelles and cannot be released by conventional organic solvents. However, hydrolysis by mineral acids followed by extraction with organic solvents has been found to be very effective tool for releasing these bioactive compounds [7].

Many analytical techniques are being applied for purification, identification and authentication of bioactive compounds present in plant extracts. However, LC/MS is very powerful analytical technique for separation, qualitative and quantitative determination of unknown medicinal compounds and their confirmation by comparing their fragmentation patterns with those of known standards. LC/MS is not only applicable to chemical analysis of plant phenolic compounds, but it also has wide applications in pharmaceutics, food, agrochemicals, industrial and environmental analysis [8].

Digera muriacata (L.) Mart. is an important medicinal herb belonging to family *amaranthaceae.* It grows throughout the south Asia. Its different parts are used medicinally. Ethnopharmacological studies demonstrated that *D. muriacata* can be successfully used in the treatment of renal disorders. It has been used to treat CCl_4-induced toxicity in kidneys and testes to investigate its medicinal properties. Constipation has been treated by leaves and shoots of this plant since ancient times. *D. muriacata* can also be used for treatment of digestive system disorders. Seeds and flowers are used to treat urinary diseases. Leaf paste is applied locally to prevent pus formation. This plant can be used to cure weak bones and treat various infections [9]. Hence, the current research project was designed to compare the different biological activities of hydrolyzed and non-hydrolyzed extracts of *D. muriacata* and to chemically characterize various bioactive compounds present in hydrolyzed extract by LC-MS/MS technique.

EXPERIMENTAL

Extraction of plant material: Fresh sample of *Digera muriacata* was collected from different rural areas of Faisalabad, Pakistan. Its leaves were manually separated, washed, shade dried and ground to fine powders. Powdered sample was then extracted with methanol/water (70 %, 50 % and 30 % v/v) to get non-hydrolyzed extracts; and acidified methanol (0.5 N, 1.0 N and 2.0 N) to get hydrolyzed extracts. Both types of extracts were concentrated under reduced pressure and stored at 4 °C until further analysis.

Mutagenic and antimutagenic activity: The mutagenic and antimutagenic potential was evaluated by Ames test following a modified procedure of Razak and Aidoo [10]. Reagent mixture was comprised of Davis-Mingioli salt, D-glucose, bromo-cresol purple, D-biotin and *L*-histidine.

Reagent mixture, herbal extract, sterile distilled water, standard mutagens and two mutant strains: *S. typhimurium* TA98 and *S. typhimurium* TA100 were mixed in several bottles at the amounts as indicated in Table-1.

The contents of each bottle (200 µL) were dispensed into separate 96-well microtitration plates using a multichannel pipette. The plates were placed in an air-tight plastic bag and incubated at 37 °C for 4 days. All plates were observed visually and purple wells were scored as negative while yellow, partially yellow or turbid wells were scored as positive (revertants).The 'blank' plate was observed first and the rest of the plates were read only when all wells in the blank plate were coloured purple indicating the assay was not contaminated. The 'background' plate (no herbal extract or standard mutagen added) showed the level of spontaneous mutation of the test bacteria. The extract was considered toxic if all wells in the test plate showed purple coloration. For a herbal extract to be mutagenic, the number of positive wells had to be more than twice the number of positive wells in the 'background' plate.

The antimutagenic effect was measured as percentage inhibition of mutagenecity by the following formula:

$$\text{Antimutagenecity (\%)} = \left(1 - \frac{\text{Revertants of plate 5} - \text{Spontaneous Revertants}}{\text{Revertants of plate 3} - \text{Spontaneous Revertants}}\right) \times 100$$

The antimutagenic effect was considered as 'strong' when percentage inhibition of mutagenecity was more than 40 %, 'moderate' when percentage inhibition was 25-40 % and 'weak' when percentage inhibition of mutagenecity was less than 25 % [11].

Chemical characterization: The various phenolic compounds responsible for different biological activities were characterized by applying LC-ESI-MS/MS technique. First of all, crude extracts were purified and concentrated by solid phase extraction (SPE) before subjecting to LC/MS analysis.

LC-ESI-MS/MS Analysis: The equipment consists of liquid chromatography coupled with mass spectrometry (LC/MS) using a ThermoFisher system in which HPLC (Surveyor) system was equipped with linear ESI-Ion Trap (LTQ XL) mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA).

In order to get chromatographic separation, 5 µL of each sample was injected through autosampler (Surveyor autosampler plus) into the HPLC system (Surveyor) equipped with reverse phase C-18 column (Phenomenex 250 mm, 5 µm particle size). After injecting the sample, elution was carried out at flow rate of 5 mL/min using gradient elution. Solvent A was prepared by water:acetonitrile:trifluoroacetic acid at

TABLE-1
SET-UP OF AMES TEST IN LIQUID CULTURE (FLUCTUATION ASSAY)

Plate No.	Treatment	Volume added (mL)				
		Mutagen standard	Herbal extract	Reagent mixture	Deionized water	*Salmonella* test strain
1	Blank	–	–	2.5	17.5	–
2	Background	–	–	2.5	17.5	0.005
3	Standard mutagens	0.1	–	2.5	17.4	0.005
4	Samples for mutagenic test	–	0.005	2.5	17.5	0.005
5	Samples for antimutagenic test	0.1	0.005	2.5	17.4	0.005

90:10:0.1 % (v/v) and solvent B at 10:90:0.06 % (v/v). Elution was performed using the following gradient: 0-10 min: 10-35 % B, 10-20 min: 35-42 % B and 20-30 min: 42-100 % B. A photodiode array was used as detector and prominent peaks were also analyzed by mass spectrometer (LTQ XL ThermoFisher Scientific). The compounds corresponding to these peaks were ionized using Atmospheric Pressure Electrospray Ionization (ESI) probe at negative ion mode. Other ESI-MS conditions included sheath gas flow rate 45 (arb) or 9 L/min, auxiliary gas flow rate 10 (arb) or 2 L/min, APCI vaporization temperature 300 °C, corona source voltage 4.5 kV, source current 4.10 µA, ion transfer capillary temperature 275 °C, capillary voltage 45 V and tube lens voltage 110 V.

Identification of phenolics was conducted under full scan mode in the range of 100-600 *m/z*. MS^2 analysis for each parent ion peak was performed at different collision induced dissociation (CID) powers. X-calibur 1.4 software was applied for calibration of MS data [7,12].

RESULTS AND DISCUSSION

Extraction is very crucial process for releasing plant secondary metabolites from plant cells. These plant secondary metabolites may serve as antioxidants, antimutagens, anticancerous and antibacterial agents [10,11]. 0.5 N acidified methanol gave maximum percentage of extract yield (36.9 ± 0.8 %) among hydrolyzed extracts while 70 % acidified methanol produced highest yield of extract (14.6 ± 0.05 %) among non-hydrolyzed extraction as presented in Table-2. Acidified methanol gave better extract yield than aqueous methanol. This marked difference in extract yields of hydrolyzed and non-hydrolyzed extracts might be due to the fact that acidic methanol was able to recover more phenolics and bioactive compounds from internal cellular organelles of plant cells [13].

Mutagenic and antimutagenic potential: Many types of inflammatory diseases, almost all types of cancers and even aging processes are caused by DNA damage. Cellular genome, everyday, undergo about one million lesions averagely; however, there is natural cellular system to repair these lesions. However, sometimes, one or two lesions remain unrepaired resulting in different types of mutations [14,15]. Many epidemiological studies have shown that consumption of fruits and vegetables lead to decrease risk of cancer and other degenerative diseases. This can be explained by considering antioxidant properties of food components such as vitamins A, C and E, phenolics, flavonoids and carotenes. Many of these antioxidant compounds result in reduction of ROS which lead to decrease in number of oxidatively modified DNA bases [16,17].

Both hydrolyzed and non-hydrolyzed extracts were found to be non-mutagenic, so they were examined for their antimutagenic potential. Table-2 represents the antimutagenic behaviour of both hydrolyzed and non-hydrolyzed extracts of *D. muriacata* as compared to standard antioxidant compounds *i.e.* ascorbic acid and gallic acid. Hydrolyzed extracts exhibited greater antimutagenic potential than those of non-hydrolyzed extracts. Moreover, both hydrolyzed and non-hydrolyzed extracts were found to be strong antimutagens as they presented quite comparable percentage antimutagenecity to standard antioxidants. This high percentage of antimutagenic behaviour might be due to high phenolic contents which are capable of interacting directly or indirectly with mutagenic agents.

LC-ESI-MS/MS analysis: The chemical characterization of bioactive compounds was carried out by LC-ESI-MS/MS analysis. Table-3 presents the results of LC-ESI-MS/MS analysis of 0.5 N acidified methanolic extract of *Digera muriacata.* In HPLC chromatogram, thirteen peaks were recorded in order of elution pattern, but ten of them were identified in the mass range of 160-600 amu. The concentrations of characterized compounds were determined from their respective intensities.

Phenolic acids and their conjugates: Peaks 1, 3, 7, 8, 9 and 10 having retention times 3.33, 4.68, 25.57, 26.45, 26.75 and 27.65 min, represent caftaric acid, rosmarinic acid, sinapic acid hexoside, 5-O-feruloylquinic acid, chebulic acid and 3,4-O-dicaffeoylquinic acid, respectively as shown in Fig. 1a.

Peak 1 corresponding to caftaric acid which is conjugate of caffeic acid and tartaric acid gave molecular ion peak at *m/z* 311.22 which on further MS/MS analysis yielded three product ion peaks; one at *m/z* 179 by loss of tartaric acid moiety, other at *m/z* 149 remained unidentified and third at *m/z* 135 which was attained by losing tartaric acid residue and CO_2 moiety from parent ion [18]. Peak 3 indicated rosmarinic acid which on MS analysis yielded $[M-H]^-$ ion at *m/z* 358.97 that was subsequently fragmented into one at *m/z* 197 by loss of caffeoyl residue and other at *m/z* 161 that corresponds to

TABLE-2
ANTIMUTAGENIC ACTIVITY (%) OF NON-HYDROLYZED AND HYDROLYZED EXTRACTS OF *Digera muriacata*

Extraction solvent	Extract yield (%)		Antimutagenic activity					
			TA98			TA100		
			A	B	C	A	B	C
		Background	8	–	–	12	–	–
		$K_2Cr_2O_7$	92	–	–	–	–	–
		NaN_3	–	–	–	94	–	–
70 % Methanol	14.6 ± 0.05		35	68.25 ± 0.69	Strong	38	64.29 ± 1.19	Strong
50 % Methanol	11.2 ± 0.07		36	67.06 ± 0.69	Strong	39	62.70 ± 0.69	Strong
30 % Methanol	8.9 ± 0.04		37	65.87 ± 0.69	Strong	40	61.90 ± 1.19	Strong
2.0 N Acidified methanol	23.0 ± 0.70		33	70.24 ± 1.19	Strong	35	67.46 ± 0.69	Strong
1.0 N Acidified methanol	30.0 ± 0.40		32	71.83 ± 0.69	Strong	34	69.44 ± 0.69	Strong
0.5 N Acidified methanol	36.9 ± 0.80		31	73.02 ± 0.69	Strong	33	70.63 ± 0.69	Strong
		Ascorbic acid	28	75.40 ± 1.37	Strong	33	73.98 ± 0.70	Strong
		Gallic acid	30	72.62 ± 2.06	Strong	36	71.95 ± 2.11	Strong

A = Revertants; B = Antimutagenecity (%); C = Antimutagenic effect.

TABLE-3
LCMS/MS PROFILE OF HYDROLYZED EXTRACT OF *Digera muriacata*

Peak No.	RT (min)	m.w.	$[M–H]^-$	MS^2 ions	Proposed compounds	m.f.	Concentration (ppm)
1	3.33	312.23	311.22	179, 149, 135	Caftaric acid	$C_{13}H_{12}O_9$	253.41
2	4.20	228.24	226.97	185, 159	Resveratrol	$C_{14}H_{12}O_3$	264.93
3	4.68	360.31	358.97	161, 197	Rosmarinic acid	$C_{18}H_{16}O_8$	255.62
4	5.47	286.24	285.12	241, 169, 151	Kaempferol	$C_{15}H_{10}O_6$	251.16
5	24.20	–	187.13	169	Unknown	–	259.11
6	24.97	–	261.15	202	Unknown	–	267.26
7	25.57	386.35	385.12	223	Sinapic acid hexoside	$C_{17}H_{22}O_{10}$	298.30
8	26.45	368.11	367.14	191, 193	5-O-Feruloylquinic acid	$C_{17}H_{20}O_9$	440.36
9	26.75	578.52	577.21	285	Kaempferol-O-dirhamnoside	$C_{27}H_{30}O_{14}$	672.10
10	27.65	516.45	515.04	353, 191, 179, 135	3,4–O-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	284.32
11	28.23	356.23	355.21	337, 249	Chebulic acid	$C_{14}H_{12}O_{11}$	328.86
12	28.64	494.14	493.20	331	Malvidin-3-O-glucoside	$C_{23}H_{26}O_{12}$	390.00
13	29.08	–	475.01	326	Unknown	–	344.29

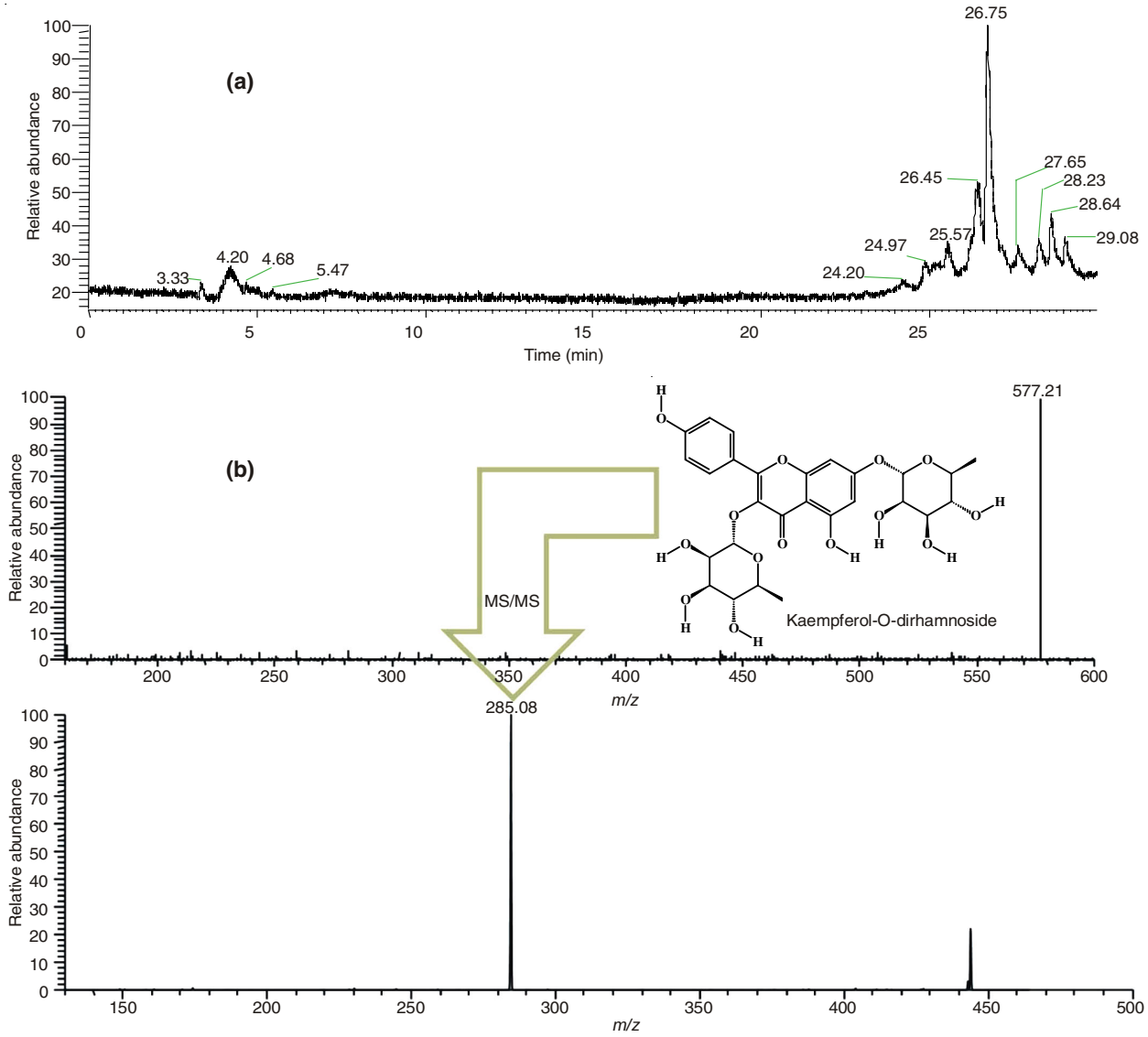

Fig. 1. HPLC chromatogram of *Digera muriacata* (a) and MS spectrum with fragmentation pattern of kaempferol-O-dirhamnoside (b)

$[caffeic\ acid\text{-}H\text{-}H_2O]^-$ [19]. Peak 7 standing for sinapic acid hexoside gave MS pseudo molecular ion peak at *m/z* 385.12. This compound was authenticated by production of MS^2 peak at *m/z* 223 that was due to removal of hexose residue [20]. 5-O-Feruloylquinic acid gave peak 8 in HPLC chromatogram that produced an intense molecular ion peak at *m/z* 367.14 and was confirmed by two daughter ion peaks. One at *m/z* 191 was produced by loss of ferulic acid residue; other at *m/z* 193 corresponded to removal of quinic acid moiety [21]. Peak 10 indicated 3,4-O-dicaffeoylquinic acid, that on subsequent

analysis by mass spectrometry gave pseudo molecular ion peak at 515.04 amu which fragmented into four peaks; one at *m/z* 353 by loss of caffeoyl residue, other at *m/z* 191 by removal of both caffeic acid moieties, third at *m/z* 179 due to elimination of caffeic and quinic acid residues and fourth at *m/z* 135 corresponding to decarboxylated caffeic acid [22]. Peak 11 of LC chromatogram (Fig. 1a) represented chebulic acid which gave an intense peak at *m/z* 355.21for deprotonated molecular ion $[M-H]^-$ that was ascertained by two daughter ion peaks; one at *m/z* 337 by loss of H_2O molecule and other at *m/z* 249 that was characteristic of chebulic acid as studied by Pfundstein *et al.* [23].

Stilbenes: Peak 2 (RT 4.20 min) corresponded to resveratrol which on MS analysis gave a deprotonated molecular ion peak at *m/z* 226.97 that was cleaved into two product ion peaks at *m/z* 185 and 159 and are characteristic of resveratrol as shown by Sun *et al.* [24].

Flavonol and flavonol glycosides: Peak 4 having retention time 5.47 min gave an intense peak at *m/z* 285.12 in mass spectrometric analysis showing the presence of kaempferol that was subsequently authenticated by three product ion peaks at *m/z* 241, 169 and 151 which were due to cleavage of heterocyclic ring of kaempferol as were matched by reference standard. Peak 9 (RT 26.75 min) yielded a deprotonated molecular ion peak at *m/z* 577.21 (Fig. 1b) which gave an indication for kaempferol-O-dirhamnoside that was confirmed by fragmented ion at *m/z* 285 corresponding to kaempferol residue by losing two rhamnoside moieties [20]. Malvidine-3-O-glucoside produced a peak in LC chromatogram designated as peak 12 (RT 28.64 min) which further produced an intense molecular ion peak at *m/z* 493.20 in mass spectrograph. This parent ion yielded a fragment ion peak at *m/z* 331 by the loss of glucose residue [18].

Quantitative analysis indicated that kaempferol-O-dirhamnoside was the most abundant of all bioactive compounds with concentration of 672.10 ppm. 5-O-feruloylquinic acid (440.36 ppm), malvidine-3-O-glucoside (390.0 ppm) and chebulic acid (328.86 ppm) were also quite abundant.

Conclusion

In the current study, hydrolyzed and non-hydrolyzed extracts of *Digera muriacata* were analyzed for their cytotoxicity by mutagenic assay, then their therapeutic values were examined in terms of antimutagenic activity. Both types of extracts were found to be non-mutagens; and hydrolyzed extracts exhibited higher percentage of antimutagenic activity than those of non-hydrolyzed extracts. Hence, optimized hydrolyzed extract was subjected to chemical characterization of medicinal compounds by LC-ESI-MS/MS analysis. It depicted the presence of high concentrations of kaempferol-O-dirhamnoside, 5-O-feruloylquinic acid, malvidine-3-O-glucoside, chebulic acid, *etc*. Thus, *Digera muriacata*can be used as a potential source of medicinal compounds in the treatment of various degenerative diseases.

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