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PHYTOCHEMICAL CHARACTERIZATION BY LC-MS AND ANTIOXIDANT EVALUATION OF MEDICINALLY IMPORTANT HERBS- POTENTIAL TARGET FOR HEALTH BENEFITS

SINGH M[1*], KUMAR P[1*] AND BALA A[1]

School of Bioengineering and Food Technology,Shoolini University, Post Box No.9, Head Post Office, Solan (HP) – 173212, India[1].

***Corresponding Authors: 1. Manjeet Singh, Research scholar; E-mail: msdogra0007@gmail.com;Phone: +91-8263052007; 2. Dr. Pankaj Kumar, Assistant Professor; Phone: +91-9736957022, Email-chauhanbiochem084@gmail.com**

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ABSTRACT

The present investigation was carried out to evaluate the phytochemical constituents, antioxidant potential and for phytochemical characterization of herb extracts of *Terminalia Arjuna, Zingiber officinale* and *Mentha arvensis* from local Himalayan region of Himachal Pradesh and also to evaluate the correlation between antioxidant activities with total phenol and flavonoids contents. The total phenolic content was found to be 191.35±3.62, 174.45±5.78 and 142.60±3.58 µg/ml gallic acid equivalents and similarly, flavonoid content was 312.26±6.24, 97.45±6.02 and 77.74±4.01 µg/ml rutin equivalents for *T. Arjuna, Z. officinale* and *M. arvensis* respectively. The antioxidant activity values of DPPH scavenging for herb extract were found to be (IC_{50}-8.18, 19.21 and 25.07 µl/ml), FRAP activity values were (IC_{50}- 8.86, 27.33 and 60.03 µM/ml Fe (II) equivalents) and Nitric oxide scavenging values were (IC_{50}- 20.87, 70.12 and 45.74 µl/ml) for *T. Arjuna, Z. officinale* and *M. arvensis* respectively. The investigation revealed significant correlation between total phenolics and flavonoid contents with antioxidant activities of herb extracts.LC-MS analysisshowed the presence of Cannabivarin, Altenusin and Acteol for *T. Arjuna*, Allomatrine and Carnosine for *M. arvensis* and Auraptenol and Absinthin for *Z. officinale.* This was the first report which provide insight into potential of these easily accessible

cradles of natural antioxidants and could be explored by the pharmaceutical, medical, and health food industries.

Keywords: Gallic acid, Rutin, Aluminum chloride, Ascorbic acid, DPPH, LC-MS.

INTRODUCTION

From the dawn of civilization, human has been utilizing the important biological belongings of various plants for the treatment of different types of the diseases. The plant and their parts which are consumed daily contain many macronutrients, micronutrients and non-nutrient compounds which play a protective role in the pathogenesis of life intimidating human diseases and disorders. Nature has conferred us with many different kinds of plants and all parts individually or totally exhibit therapeutic properties showing different biological bustle and antioxidant potency [1, 2]. The phenolic compounds or polyphenols, secondary vegetal metabolites, constitute a wide and complex array of phytochemicals that exhibit antioxidant action and consequently a beneficial physiological effect [3] and act as a prophylactic agent, which has motivated research into food science and biomedicine [4].Processed foods contain significant amounts of polyunsaturated fatty acids (PUFAs) and it is necessary to add antioxidants to prevent oxidation, to increase product shelf life, decrease rancidity, discoloration etc. synthetic antioxidants like butylatedhydroxyasinole (BHA), butylatedhydroxytoluene (BHT) and propyl gallate (PG) are widely used in the food industry but it has raised serious objections because of their toxic nature, carcinogenetic and low solubility [5]. Because of these detrimental consequences, the reduction of free radicals or reactive oxygen species in both human and food system is highly desirable. Hence the investigators have turned their attention to target and identify alternative novel antioxidants from natural sources which have therapeutic properties, lesser side effects and cost effective as compared to synthetic ones. *Terminalia arjuna* (TA) belonging to the family Combretaceae, is an important medicinal plant widely used by the tribal and rural people for alleviating angina and other cardiovascular conditions for over three centuries in India. Experimental studies have revealed that the stem bark exerts significant inotropic and hypotensive effect, increasing coronary artery flow and protecting myocardium against ischemic damage [6]. *Zingiber officinale* belongs to the family Zingibeaceae and is widely used by the people for treating diabetes, high blood pressure, cancer, fitness and many other illnesses [7]. Mint (*Mentha arvensis*) is a genus of flowering plants in the family Lamiaceae. It is well known for its properties related to indigestion, stomach cramps, menstrual cramps, flatulence, upset stomach, nausea, vomiting, and colic in children. It is also a source of effective antioxidants [8]. The focus of present study is the virtual evaluation of phytochemical constituents, antioxidant potential and phytochemical characterization in medicinal herbs as source for human health.

MATERIALS AND METHODS

Chemicals and Reagents

Ascorbic acid, aluminum chloride, 2,2-diphenyl-2-picrylhydrazyl (DPPH), Sodium nitrite ($NaNO_2$), 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co., U.S.A. Ferric chloride, Folin-Ciocalteu reagent, Gallic acid and Rutin were procured from Loba Chemie Pvt. Ltd, Mumbai, India. All the chemicals and reagents used in this study were of analytical grade.

Collection of plant material

Leaves, bark and rhizomes of *M.arvensis, T. arjuna* and *Z.officinale* were collected from local region of Solan, Himachal Pradesh, India. The plant materials were washed thoroughly with tap water and then surface sterilized with 70% ethanol.

Preparation of extract:

Aqueous extract of *Terminalia arjuna*: *T. arjuna* bark sample was dried in vaccum oven at 60°C, ground in a laboratory mixer grinder for 50 sec. Powder sample of 10 gm was mixed with deionized water (1:10 ratio) and was shaken in orbital shaker at 350 rpm and 40°C for 24 hours and the supernatant was filtered and the supernatant was centrifuged at 2500 rpm, and stored at refrigeration condition for future use.

Preparation of Ginger extracts: Fresh rhizomes of ginger were cut into thin slices (2 mm thickness). Water extracts of ginger were prepared by blending 50 g of sliced ginger in 250ml of deionized water using a laboratory blender at a maximum speed for 2 min. The water extract of ginger was sonicated at 50 °C for 15 min and filtered through cheese cloth. This water extract of ginger was pasteurized and stored in sterilized glass bottles at 4 °C.

Extraction of Mint juice: The fresh leaves of Mint were washed properly and blended in a laboratory blender to paste with deionized water (1:5). The water extract of Mint was sonicated at 50 °C for 15 min and filtered through muslin cloth to obtain the juice extract. This water extract of Mint was pasteurized and stored in sterilized glass bottles at 4 °C.

Determination of total phenolic contents: The amount of total phenolic compounds in studied samples was determined according to the Folin-Ciocalteu procedure [9]. Total phenolic content was calculated from calibration curve of gallic acid (10-320μg/ml) and expressed in terms of gallic acid equivalents (GAE) per ml of sample.

Determination of total flavonoid Content: The total flavonoids content in studied samples was determined by using aluminium chloride ($AlCl_3$) method [10]. The flavonoid content was calculated from standard curve of rutin (20-100 μg/ml) and expressed as rutin equivalents (RE) per ml of sample.

***In-vitro* antioxidant activity**

DPPH radical scavenging activity: DPPH radical scavenging activity of the samples was measured by modified method [11].

Ferric Reducing Antioxidant Power (FRAP) assay: The ability to reduce ferric ions was dignified using the methoddescribed by Benzie and Strain [12]. Ascorbic acid was used as positive reference standard. The antioxidant capacity based on the ability to reduce ferric ions of samples was calculated from the linear calibration curve of $FeSO_4$ (2.5-20μM) and expressed as μM $FeSO_4$ equivalents per ml of sample.

Nitric oxide (NO) scavenging assay: Nitric oxide scavenging assay was carried out using sodium nitroprusside method as described by Sreejayan and Rao with ascorbic acid as positive standard [13].

Liquid Chromatography-Mass Spectroscopy (LC-MS): LC-MS/MS analysis was performed on an Agilent 6410 LC/MS-MS (Agilent Technologies, Santa Clara, CA, USA) triple-stage quadrupole mass spectrometer equipped with an ESI interface and liquid chromatography analyses were carried out using an Agilent 1260 Infinity (Agilent). Analytical chromatographic separations of sample extracts were carried out on a chromolith performance RP-18e column (4.6 × 100 mm; Merck) protected by a chromolith guard column from the same company. The column temperature was maintained at 30 °C and analysis was carried out at a wavelength of 259 nm. The chromatographic separation was performed at a flow rate of 0.5 $mLmin^{-1}$ and the sample injection volume 5 μl under a gradient program in which eluent A composed of 0.1 % Formic acid in Water and mobile phase B Composed of Acetonitrile .The gradient system was applied as follows 5-60%of B for 0-18 min, then hold 60% of B for 18-25 min, 60-5% of B for 25-27 min and for 3 min at initial composition of mobile phase. The total analysis run time was 30 min. The following parameters were used throughout the MS experiment: for electro spray ionization with positive ion polarity the capillary voltage was set to 3KV, the capillary temperature to 300°C, the nebulizer pressure to 35 psi, and the drying gas flow rate to 12 $Lmin^{-1}$ [14].

Statistical analysis: Each sample was analyzed individually in triplicates and the results are expressed as the mean value (n = 3) ± standard deviation. Antioxidants assay were analyzed by using two way ANNOVA in Graph Pad Prism 5 software. The correlation coefficients between studied parameters were demonstrated by linear regression analysis.

RESULTS AND DISCUSSION

Determination of total phenolic contents (TPC)

The total phenolic content of aqueous extract of *T. Arjuna, Z.officinale* and *M.arvensis* was determined by using Folin-Ciocalteu method. The phenolic content was calculated from standard curve of gallic acid (standard plot: y=0.0042x, R^2=0.9932) **(Fig.1)**. The total phenolic content was found to be 191.35±3.62, 174.45±5.78 and 142.60±3.58µg/ml gallic acid equivalents for *T. Arjuna, Z.officinale* and *M.arvensis* respectively. Aqueous extract of *T. Arjuna* (191.35±3.62 µg/ml gallic acid equivalents) possess higher amount of total phenolic content as compared to that of *Z.officinale* and *M.arvensis* extracts.

Determination of Total Flavonoid Content (TFC)

The total flavonoids content of aqueous extract of *T. Arjuna, Z.officinale* and *M.arvensis* was determined by using aluminium chloride ($AlCl_3$) method. The flavonoids content was calculated from standard curve of rutin (standard plot: y=0.0043x, R^2=0.9946) **(Fig.2)**. Aqueous extract of *T. Arjuna* (312.26±6.24 µg/ml rutin equivalents) possess higher amount of total flavonoid content as compared to that of *Z.officinale* and *M.arvensis* (97.45±6.02 and 77.74±4.01µg/ml rutin equivalents).

In-vitro antioxidant activity

There are different methods to evaluate antioxidant characteristics of plants but none of them alone cannot be used for evaluating antioxidant property of extracts. Therefore, it is necessary to characterize the extract by different antioxidant mechanism.

Scavenging activity on 2, 2-diphenyl-1-picrylhidrazyl (DPPH) radical

DPPH is a stable free radical that accepts an electron or hydrogen radical from antioxidants present in the samples to be analyzed and become a stable diamagnetic molecule and is widely used to assess the radical scavenging activity of antioxidant compounds [15]. The percentage scavenging activity of the analyzed aqueous extracts were determined using different concentrations (2.5-20 µl/ml) which have been taken to compare with the standard ascorbic acid. The DPPH radical scavenging capacity in this study has been reported after 30 minutes of incubation for all samples analyzed. *T. Arjuna*aqueous extract is the only sample which showed observable antioxidant activity in all different concentrations analyzed. Extent of DPPH radical scavenged was determined by increase in intensity of violet color in the form of IC_{50} values, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in specified time period. $IC_{50}$of the all methanolic extracts along with standard ascorbic acid has been shown in **Fig 3**. IC_{50} value of *T. Arjuna*extract (8.18µl/ml) is lower than that of *Z.officinale* and *M.arvensis* (19.21 and 25.07 µl/ml) whereas ascorbic acid has IC_{50}-14.52µl/ml.The results revealed that aqueous extract of *T. Arjuna* is a more effective scavenger than the aqueous extract of *Z.officinale* and *M.arvensis.*

Ferric Reducing Antioxidant Power (FRAP) assay

Frap assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+}- TPTZ] complex and producing a colored ferrous tripyridyltriazine [Fe^{2+}-TPTZ]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. Hence the FRAP assay provides a direct estimation of the level of antioxidants or reductants present in a sample. The IC_{50} values were found to be 6.13 µM/ml, 8.86, 27.33 µM/ml and 60.03 µM/ml Fe (II) equivalents for ascorbic acid, *T. Arjuna, Z.officinale* and *M.arvensis* extract respectively. The results obtained in this study are confirmed the antioxidant activity for these aqueousextracts and it is clear that existence of discrete differences between the extracts in acapacity of reducing power **(Fig-4)**.

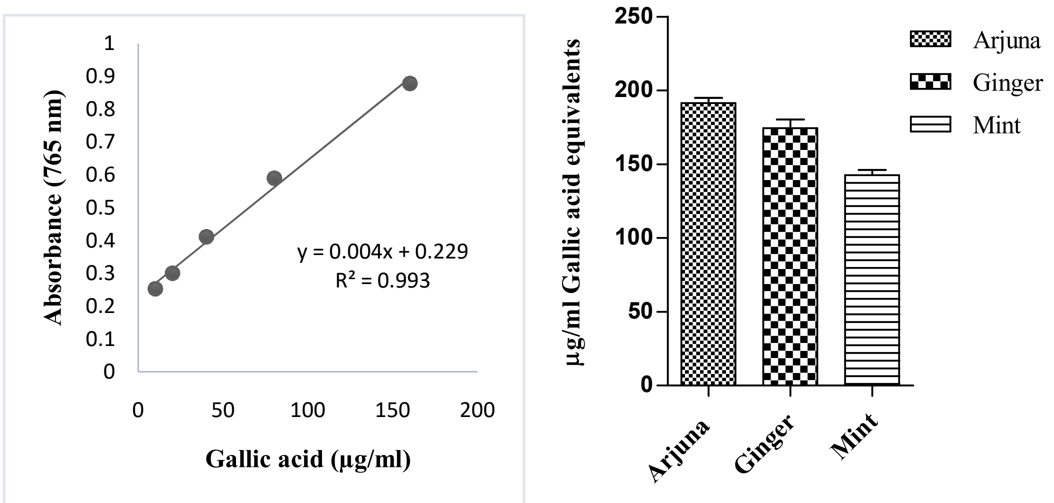
Nitric oxide scavenging assay

Nitric oxide radical is well known as it has an important role in various types of inflammatory processes. The production of nitric oxide radical at a sustained levels result in direct tissue toxicity and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis [16]. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite.Aqueousextract also moderately inhibits nitrite formation by directly competes with oxygen to react with nitric oxide. The scavenging of NO by the aqueous extract was increased in concentration dependent manner. The aqueous extract of *T. Arjuna* showed maximum activity of 66.37% at 25 µl/ml as compared to that of *Z.officinale*, *M.arvensis* and ascorbic acid at the same concentration exhibited 17.51%, 28.97 %and 40.61% inhibition respectively **(Fig-5)**. IC_{50} values were found to be 30.11 µl/ml, 20.87 µl/ml, 70.12µl/ml and 45.74µl/ml for ascorbic acid, *T. Arjuna*, *Z.officinale* and *M.arvensis* extracts respectively**.**

Correlation of total phenolic content and flavonoid content with antioxidant activity

There are many correlation studies which demonstrate a link between antioxidant activities in plants and their phenolic and flavonoid contents, underlining the significant contribution which phenolics and flavonoids make to antioxidant activities [17]. Our findings also showed that total phenolics and flavonoids were directly correlated with antioxidant capacity of *T. Arjuna*, *Z. officinale* and *M. arvensis* herb extracts as shown in table-1.

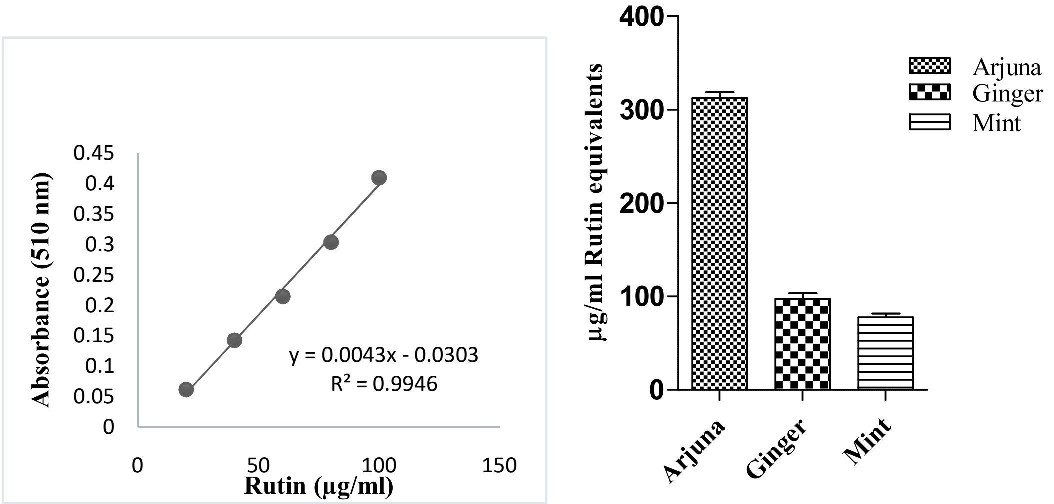
Analysis using Liquid chromatography-mass spectrometry (LC-MS): The Liquid Chromatography Mass Spectroscopy (LC-MS) of methanolic bark extract of *Terminalia Arjuna* shown in **Fig 6.1**showed molecular peak at m/z 283.00 is good agreement with empirical formula $C_{19}H_{22}O_2$ i.e. Cannabivarin. The compound showed molecular peak at m/z 291.00 is good agreement with empirical formula $C_{15}H_{14}O_6$ i.e. Altenusinas shown in **Fig 6.2**. The mass spectrum showed the molecular peak at m/z 503.30 is good agreement with empirical formula $C_{30}H_{46}O_6$ i.e. Acteol as shown in **Fig 6.3**. The methanolic leaf extract of *Mentha Spicata* shown in **Fig 7.1** showed molecular peak at m/z 173.10 is good agreement with empirical formula $C_{15}H_{24}N_2O$ i.e. Allomatrine. The compound showed molecular peak at m/z 249.20 is exactly in agreement with the empirical formula $C_6H_{12}N_4$O2i.e. Carnosine as shown in **Fig 7.2**. The methanolic rhizome extract of *Zingiber officinale* shown in **Fig 8.1** showed molecular peak at m/z 261.30 is good agreement with empirical formula $C_{15}H_{16}O_4$ i.e. Auraptenol. The compound showed molecular peak at m/z 496.70 is good agreement with the compound Absinthin i.e. $C_{30}H_{40}O_6$as shown in **Fig 8.2**.



A) B)

Fig 1(A-B)

Fig-1: Total phenolic content in herb extracts of *T. Arjuna*, *Z. officinale* and *M. arvensis* shown in B) and Standard curve of gallic acid shown in A). Phenolic content of herb extracts represented as µg/mlgallic acid equivalents. Values are expressed as mean ± standard deviation (n = 3).



A) B)

Fig 2(A-B)

Fig-2: Total flavonoid content in herb extracts of *T. Arjuna*, *Z. officinale* and *M. arvensis*shown in B) and Standard curve of rutin shown in A). Flavonoid content of herb extracts represented as µg/ml rutin equivalents. Values are expressed as mean ± standard deviation (n = 3).

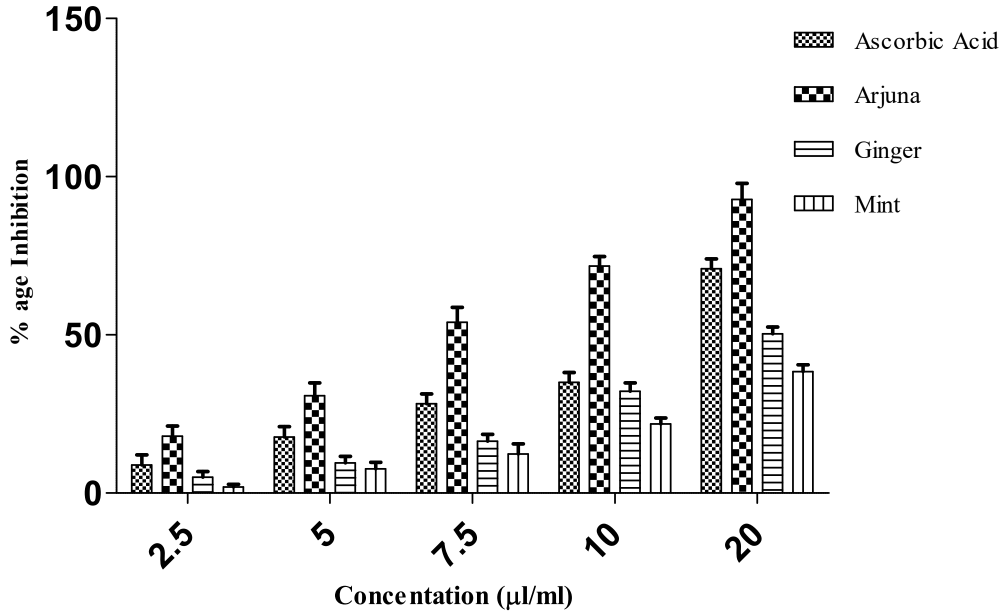


Fig 3

Fig 3- Showing DPPH radical scavenging activity of herb extracts of *T. Arjuna*, *Z. officinale* and *M. arvensis* in concentration dependent manner with standard ascorbic acid. Values are expressed as mean ± standard deviation (n = 3).

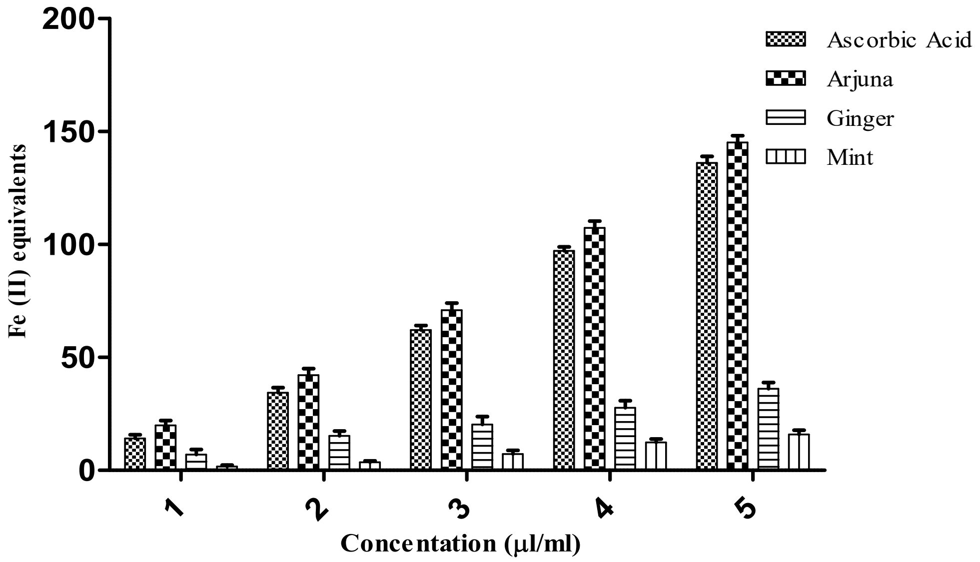


Fig 4

Fig 4-Ferric reducing antioxidant power of herb extracts of *T. Arjuna*, *Z. officinale* and *M. arvensis*in different concentrations with standard ascorbic acid. Values are expressed as mean ± standard deviation (n = 3).

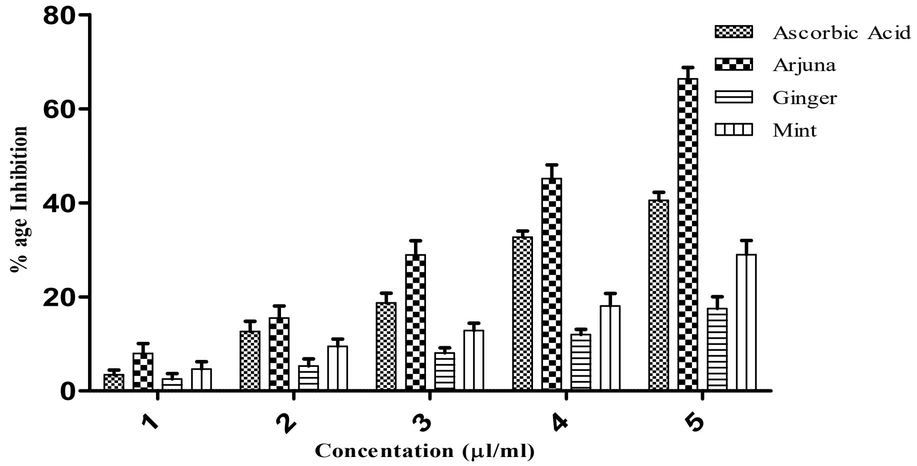


Fig 5

Fig 5- Nitric oxide scavenging activity ofherb extracts of *T. Arjuna*, *Z. officinale* and *M. arvensis* in concentration dependent manner with standard ascorbic acid. Values are expressed as mean ± standard deviation (n = 3).

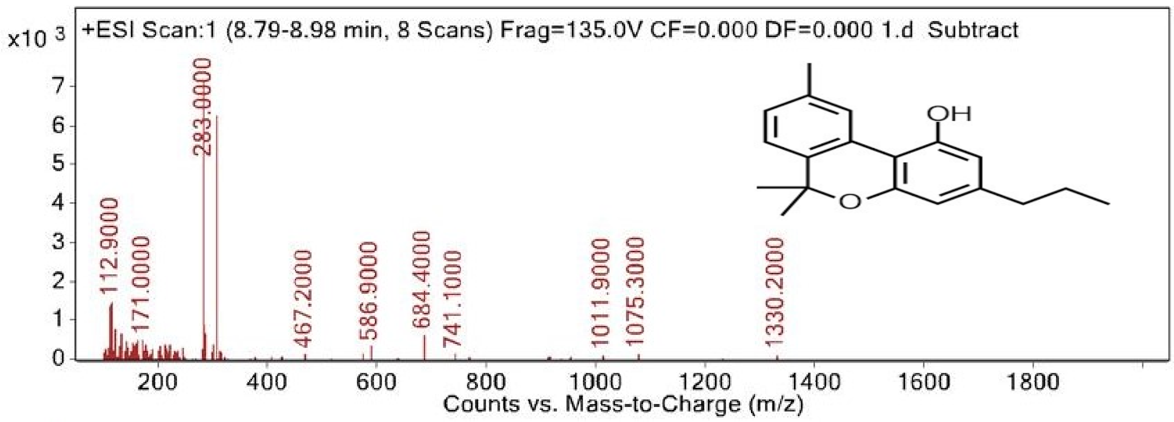


Fig 6.1. The MS spectra of Cannabivarin in positive mode(m/z=283.0000,t_R =8.88) and its structure for identication($C_{19}H_{22}O_2$).

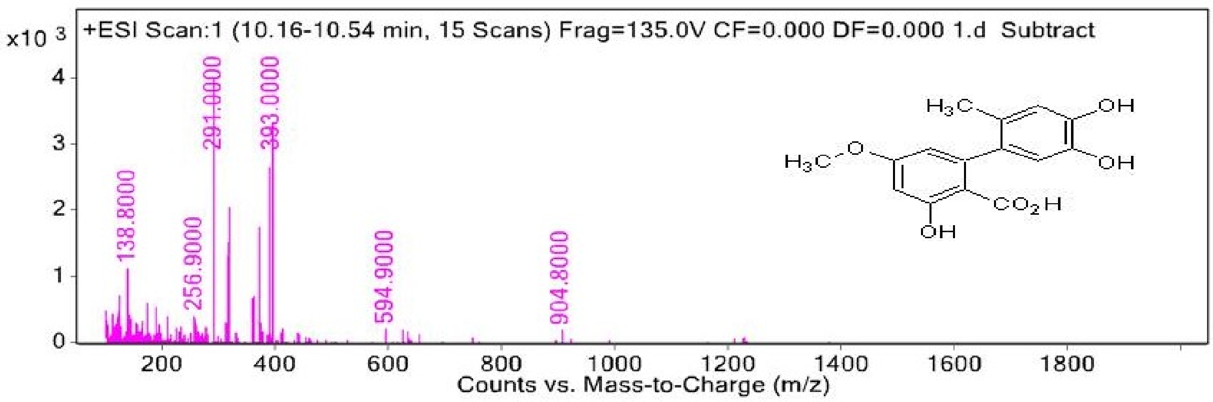


Fig 6.2. The MS spectra of Altenusin in positive mode (m/z=291.0000,t_R =10.36) and its structure for identication ($C_{15}H_{14}O_6$).

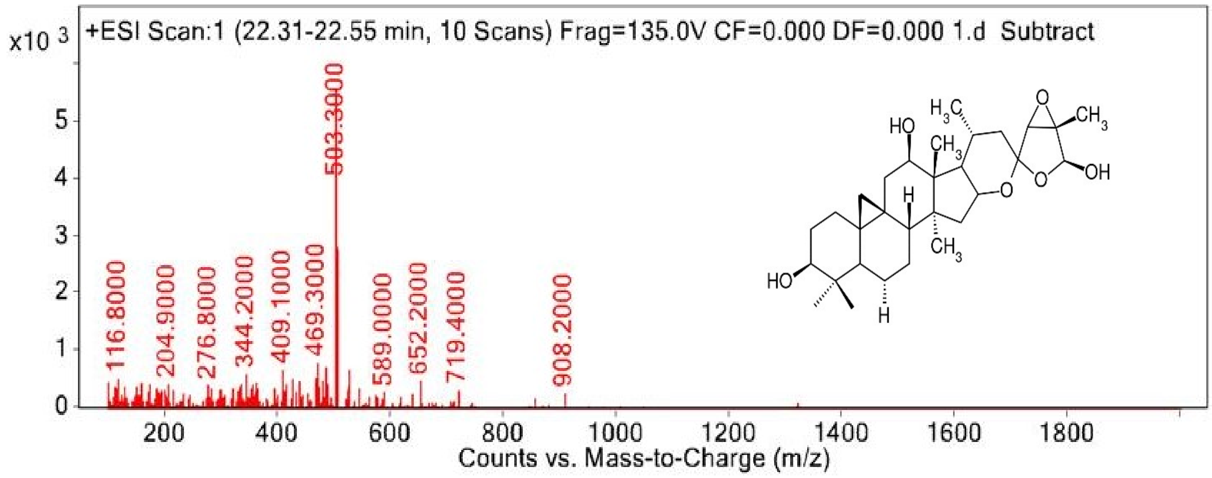


Fig 6.3.The MS spectra of Acteol in positive mode (m/z=503.3000, t_R =22.45) and its structure for identification ($C_{30}H_{46}O_6$).

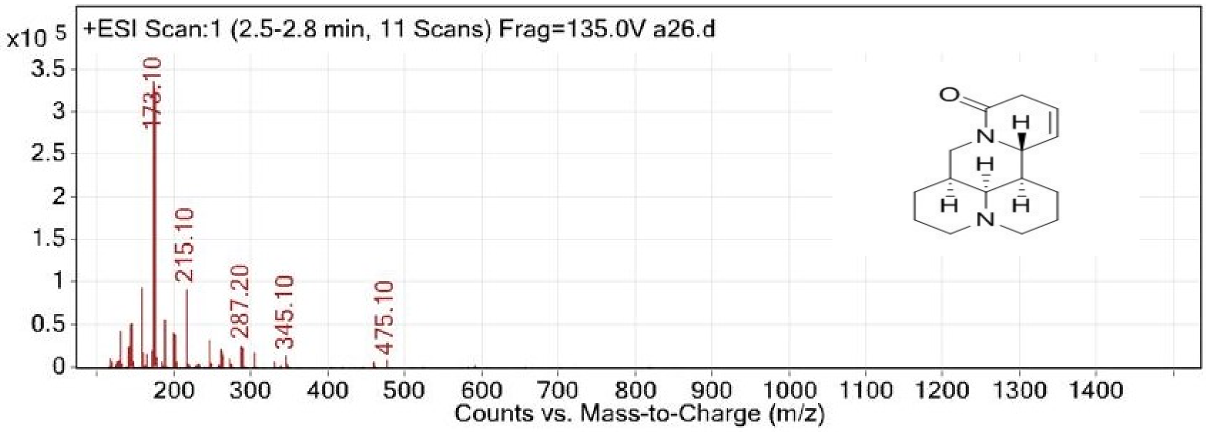


Fig 7.1.The MS spectra of Allomatrine in positive mode (m/z=173.10, t_R =2.7) and its structure for identification ($C_{15}H_{24}N_2O$).

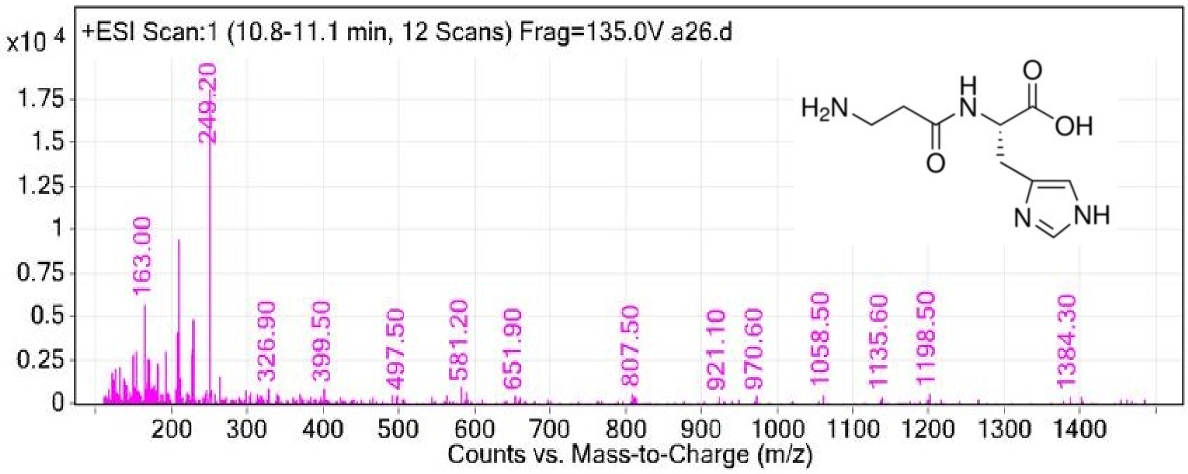


Fig 7.2. The MS spectra of Carnosine in positive mode (m/z=249.20, t_R =10.9) and its structure for identification ($C_6H_{12}N_4O2$).

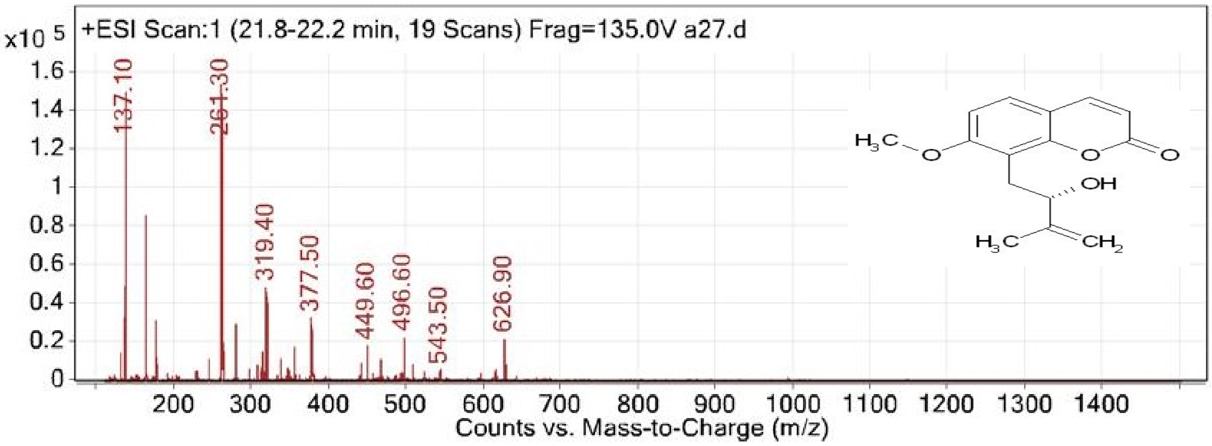


Fig 8.1. The MS spectra of Auraptenol in positive mode (m/z=261.30, t_R =21.9) and its structure for identication ($C_{15}H_{16}O_4$).

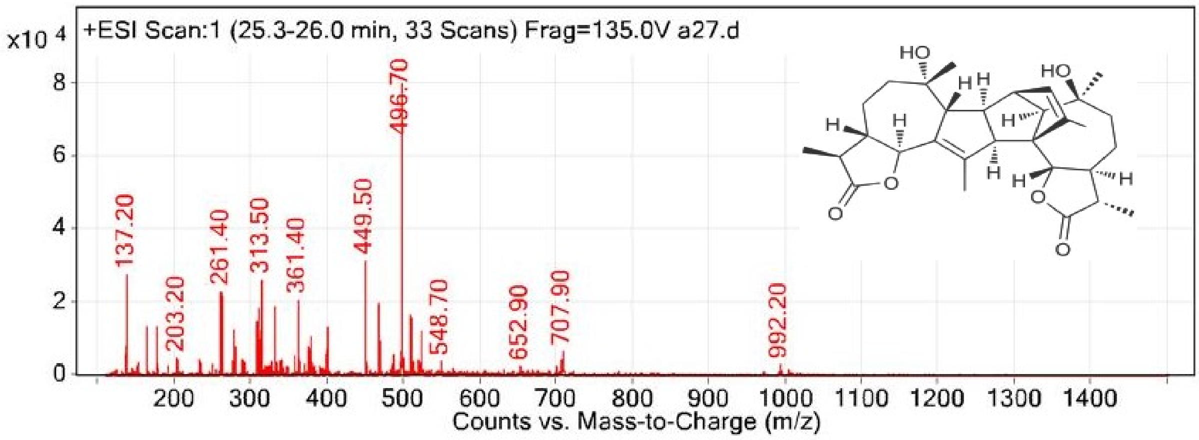


Fig 8.2.The MS spectra of Absinthin in positive mode (m/z=496.70, t_R =25.9) and its structure for identification ($C_{30}H_{40}O_6$).

Table-1- Correlation between phenolics and flavonoids with antioxidant activities of herb extracts *T. Arjuna*, *Z. officinale* and *M. arvensis.*

Antioxidant Assays	Correlation coefficient (R^2)					
	Total Phenolics			Total Flavonoids		
	Arjuna	Ginger	Mint	Arjuna	Ginger	Mint
DPPH radical scavenging activity	**0.9312**	**0.9313**	**0.9741**	**0.9693**	**0.9124**	**0.9760**
Nitric oxide scavenging activity	**0.9768**	**0.9557**	**0.9343**	**0.9957**	**0.9462**	**0.9321**
Ferric Reducing Antioxidant Power (FRAP) assay	**0.9642**	**0.8981**	**0.9931**	**0.9923**	**0.8890**	**0.9843**

CONCLUSION

The present study has contributed to considerate the holistic and the impending applications of *T. Arjuna*, *Z. officinale* and *M. arvensis* as a source of phytonutrients and antioxidants based on both TPC and TFC values along with their free radical scavenging activities and IC_{50} values. In this study, we also concluded that the aqueous extracts of the tested plants have higher phenolic and flavonoid contentand may be used for scavenging the free radicals.The results attained from various assays exhibited high antioxidant activity of the aqueous extracts even at lower concentrations, which in accordance with good correlation to total phenolic and flavonoid content. Therefore, the eminence of the phytochemical constituents and antioxidant activities of these plants in the maintenance of wellbeing is reinforced as trend of the forthcoming and can be used as an easy accessible source of natural antioxidants and therapeutics in pharmaceutical and medical diligences.

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