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Research Article

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Nutraceutical Evaluation of Horse Gram (*Macrotyloma uniflorum*) Cultivated in High Altitudes of Uttarakhand Himalaya, India

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ABSTRACT

Complete exploration of nutraceutical properties of Macrotyloma uniflorum by liquid chromatography mass spectrometry (LC-MS) and proximate analysis was performed. Seeds of M. uniflorum were collected from local farmers from the higher altitude (2178m, N-29°27.034'; E-079°46.210'). Three types of extracts were prepared with the help of soxhlet extractor and dried in vacuum dryer. The above extracts were examined by LC-MS (TOF/Q-TOF Mass Spectrometer) for the presence of alkaloids, glycosides, saponins, phenolic compounds, tannins followed by Macronutrients, micronutrients and proximate analysis followed by LC-MS analysis in Hydrolic (66), Ethanolic (66) and Methanolic extract (119). The maximum number of compounds was found in methanolic extract, compounds common in all solvents were (32), common in methanolic and ethanolic were (17) and common in methanolic and hydrolic were (11) respectively. The unique compounds found in methanolic; ethanolic and hydrolic were 58, 17, 23 respectively. Sizable numbers of metabolic compounds were found in all extracts those are considered as very useful compounds in various metabolic regulations specifically in anti-lithiasis, anti-obesity, anti-diabetes, Alzheimers disease and many other neurological disorders. Present investigation is very significant in terms of exploring the nutraceutical properties of M. uniflorum and the outcome of this study would be of wide interest to farmers and researchers working on nutraceuticals including commercial explorations.

Keywords: *LC-MS, Proximate analysis, Anti-lithiasis, Anti-obesity, Anti-diabetes, Macrotyloma uniflorum.*

INTRODUCTION

Macrotyloma uniflorum popularly known as horse gram is an annual herb that attains height up to 30 to 40 cm (Neelam et al., 2014). As an edible crop, horse gram (HG) is an excellent source of protein, carbohydrates, dietary fibre and micronutrients (Sangita et al., 2004). In traditional knowledge system horse gram is also known to have many therapeutic effects (not scientifically proven) though it has been recommended in ayurvedic medicine to treat renal stones, piles, oedema, etc (Mohar et al., 2013).

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HG is a real super food in terms of nutraceuticals. Super food refers to an extraordinary class of nutrient-dense foods having high antioxidants and protein Mehra, A. (2013). In fact, the protein content of HG is higher than most of pulses. HG is a type of slowly digestible starch ideal for diabetic and obese individuals; it suppresses the appetite and stabilizes the blood sugar level. In recent years, isolation and utilization of potential antioxidants from legumes including horse gram has achieved high attention as it decreases the risk of intestinal diseases, diabetes, coronary heart disease, prevention of dental caries etc (Mohgana et al., 2017, Saroj & Manoj, 2015). The investigations are still on to decipher the horse gram with reference to its functional molecules those directly affect the disease. The present study was designed for identification of specific functional molecules using computational biology tools. These investigations would be of great interest to develop hypothesis and design transgenics for detailed investigations. Keeping in view the increasing demand of food having therapeutic values, the present investigation definitely attribute recent scientific knowledge towards the possibilities of exploring the HG, as a source of food and nutraceuticals compounds.

MATERIALS AND METHODS

Plant Materials: Cultivars of *M. uniflorum* were collected from local farmers from Kumaun region of Mornolla (Almora) at 2178m of Uttarakhand (**Table.1**) during the harvesting seasons. All chemicals used in the investigation were of molecular biology grade.

Nutritional analysis:

Various parameters of horse gram were analyzed as per standardized procedures and protocols (**Table 2)** with minor modifications.

Preparation of Extracts:

Dried Seed material was crushed **(Figure 1)**, using mortar pestle, 20g material was used sufficient to fill the porous cellulose thimble (in a 25- x 80-mm). The solvent was heated on mantle and evaporated using soxhlet apparatus (Supertech Pvt. Ltd.) and condensate dripping into the reservoir containing the thimble with a run time of 12 hours. Aqueous extract was prepared with D.M. water (Product Code: 025600) procured from Central Drug House (P) Ltd. New Delhi. Two types of Alcoholic extracts (Ethanolic and Methanolic) were prepared with 80% ethanol and 80% methanol (procured from MOLYCHEM Mumbai). Once the process completed the alcohol and water was evaporated leaving a small yield of extracted seed material (about 2 to 3 ml) in the glass bottom flask.

LC-MS analysis of extracts:

All extracts were sent to APS Lab, Pune (Maharashtra) for analysis with the TOF/Q-TOF Mass Spectrometer (Component Model G6540B). Using standard protocols of LC-MS the bio-molecules such as carbohydrate, steroid, tannins, phenol, protein, amino acid, alkaloids, glycosides, flavonoids and saponins were identified **(Appendix-1).**

***In-silico* analysis of phytochemicals:**

Bioinformatics online databases PubMed, Pubchem and PDB were used for Structure and function identification of phytochemicals after LC-MS results. PubChem database provides information on the biological activities of small molecules. PubChem also provides a fast chemical structure similarity search tool. The Protein Data Bank (PDB) is a repository for the 2-D structural data of large biological molecules, such as proteins and nucleic acids. Structures of molecules were analyzed with online web tool http://www.chemspider.com.

Statistical Analysis:

All values were recorded as mean ± standard deviation (SD). Microsoft Excel and online graph pad software were used to statistically analysis for present investigations.

RESULTS

Nutritional analysis:

The present investigation on HG was an attempt to explore the nutraceutical value in special. Sample was selected on the basis of evaluation and protein content. Significant micro and macro nutrients found in proximate analysis (**Table 3**). Total 199: 66: 66 components found in Methanolic: Ethanolic:

Hydrolic extracts proceeded in LC-MS analysis (Complete data available on demand). Approximately 30-35 components were found useful for human health the findings were compared with the previous investigations substantiating the present findings (**Table 4**). The proximate composition analysis of the selected HG seed is presented in **Table 3**. The moisture content of the seeds was generally low, 10.80% required for safe storage limit for plant food materials. The total ash content was measured 4.26% of the total mineral content of a material. The value of crude protein and fibre content for horse gram seeds was 31.57% and 7.38%.

LC-MS analysis:

After chromatographic (LC-MS) analysis it was found that HG has many useful components those may directly or indirectly play a major role in curing certain metabolic disorders in human. A metabolic disorder can happen when abnormal chemical reactions in the body alter the normal metabolic processes. Major components (30) those found after LC-MS are given in **Table 4.** The maximum number of compounds found in methanolic extract. Compounds common in all extract; methanolic and ethanolic; methanolic and hydrolic were sequentially 32, 17 and 11. Sole compounds in methanolic; ethanolic and hydrolic were sequentially 58; 17; 23. All compounds have positive polarity; the metabolic compounds found in all extracts are of great importance in many metabolic disorders like lithiasis, obesity, diabetes, alzheimer's disease and neurological disorders. The spectral peak of specific compounds indicates a valuable amount of that compound in HG (**Figure 4**). The anti-diabetic compound Syringic acid hexoside was found in significant amount in contrast of their peak in histogram of LC-MS. Secondly Tyramine which is very important in regulation of blood pressure was also found in significant peak and main component (Saroj & Manoj, 2015) - Gingerol which is quite useful in anti-tumorigenic activity. Rutaecarpine which is useful in exerting protective effects as established in cardiac anaphylactic injury was also detected in significant amount. The functions of all components with their function are summarized (**Table 4)**. Proline-betaxanthin having detoxifying activity by which they help to regulate body temperature and pain also found significantly in extract of HG and 3D structures of significant compound for interaction with other drugs may be useful for multi targeting drug (**Figure 5**).

DISCUSSION

In the present study, biochemical screening of seeds of *M. uniflorum was* performed with aqueous and alcoholic solvents, LC-MS analysis and the nutraceutical efficacy of the seeds was evaluated. Horse gram has been documented as potential source of protein and other nutrients (Bhartiya et al., 2015). In a study Meyer et al., (2000) observed that the people who consume lofty amounts of insoluble fiber (more than 17 g/day) or cereal fiber (more than 8 g/day) had less risk of type II diabetes than people who had lower intakes, while soluble fiber intake was not allied with diabetes risk (Meyer et al., 2000). This is a clear indication that HG is a good source of fibre and protein as per the present findings. *M. uniflorum* is one of the lesser known beans. Raw *M. uniflorum* seed is rich in polyphenols, flavonoids, proteins and major antioxidants

The seeds of HG have the ability to reduce postprandial hyperglycemia by slowing down carbohydrate digestion by reducing insulin resistance, inhibiting protein-tyrosine phosphatase 1 beta enzyme (Mallikarjun, 2013). LC-MS has become one of the most widely used chemical analysis techniques because more than 85% of natural chemical compounds are polar and thermally labile as GC-MS cannot process these samples (Pitt James, 2017). In addition nutritional importance of HG has also been linked to reduced risk of various diseases due to presence of non-nutritive bioactive substances. These bioactive substances such as phytic acid, phenolic acid, fiber, enzymatic/proteinase inhibitors have significant metabolic and/or physiological effects (Prasad & Singh, 2015).

The findings of this study prove significant role of compounds present in horse gram in various human physiological disorders and summarizes a range of medicinal

properties and nutritional efficiency of HG used to treat various diseases. HG was commonly used by farmer community and stumpy profits group people due to its unacceptable taste and flavor in earlier days (Vandarkuzhali & Sangeetha, 2015). As per Charak Samhita, the seed of HG are useful in curing piles, hiccup, abdominal lump, bronchial asthma and Sushruta Samhita says that the seed powder is useful in stopping excessive perspiration (Pati & Bhattacharjee, 2013). The extract of HG exerts a Hypolipidemic and Hypoglycemic actions (Senthil, 2009) It has been also established that it is quite useful in urinary dilemma, acid peptic disorder (gastritis), constipation, sun-burn, kidney stone, female diseases (leucorrhoea, menstrual troubles, bleeding during pregnancy, post partum excessive discharges), colic caused by wind, rheumatism, hemorrhagic disease, intestinal worms etc (Prasad & Singh, 2015). The results and findings of the present study revealed and strongly support that the compounds found after LC MS analysis are very useful in anti-lithiasis, anti-obesity, anti-diabetes, in alzheimer's disease and neurological disorders with many other unknown benefits that could be an additional aspect of investigation.

Table 1: Horse gram cultivar from Kumaun region of Uttarakhand based on specific morphology, protein content and higher elevation[7]

Location	Division and Elevation	Local Name	Seed colour	Cultivar
Mornolla, Almora.	Kumaun Elevation: 2178m N-29°27.034' E-079°46.210'	Gahat	Black	SPNP-4

Table 2: Biochemical parameters and protocols used in analysis of *M. uniflorum*

S. No.	Parameters	References for protocol
1.	Proximate principles	**(8; 9)**
2.	True protein	**(10)**
3.	*In vitro* digestibility (DM/OM)	**(11)**
4.	Silage analysis (pH, moisture, lactic acid, NH_3-N)	**(12)**
5.	Calcium	**(13)**
6.	Phosphorus	**(14)**
7.	Magnesium	**(15)**
8.	Copper	**(16)**
9.	Cobalt	**(16)**
10.	Iron	**(17)**
11.	Manganese	**(15; 16; 18)**
12.	Zinc	**(15; 16; 18)**

Table 3: Proximate, micro and macro nutrients analysis

S. No.	Constituents	Value (% on DM bases)
Proximate principle analysis		
1.	Moisture	10.80%
2.	Crude Protein	31.57%
3.	Crude fibre	7.38%
4.	Ether Extract	0.82%
5.	Total Ash	4.26%
6.	Acid Insoluble ash	0.34%
Digestibility analysis		
7.	True protein	14.91%
8.	*In vitro* digestibility (Dry Matter)	77.95%
9.	Silage analysis (pH)	6.44
10.	NDF	13.14%
11.	ADF	7.35%
12.	Lignin	3.11%
Micro and macro nutrients		
13.	Calcium	1.36 %
14.	Phosphorus	0.48 %
15.	Magnesium	0.05 %
16.	Copper	14.0 ppm
17.	Cobalt	Not detected
18.	Iron	75.4 ppm
19.	Manganese	31.8 ppm
20.	Zinc	21.2 ppm

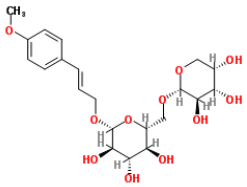
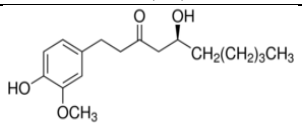
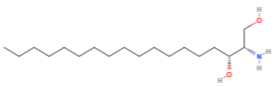
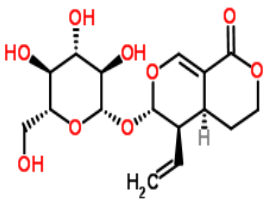
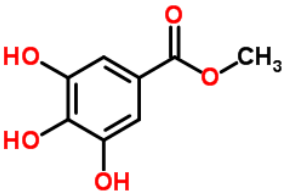
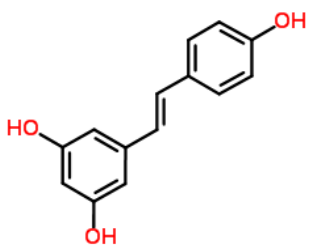
Table 4: Major components after LC-MS analysis and their metabolic function. (Molecular mass and structure were constructed with the help of https://www.webqc.org/mmcalc.php)

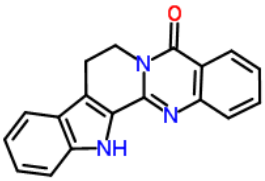
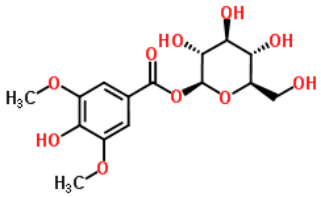
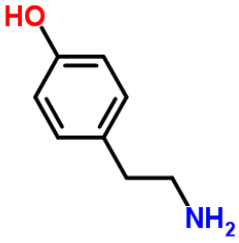
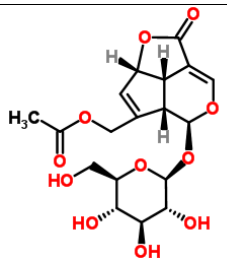
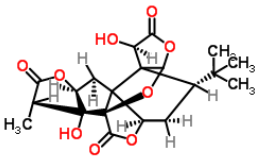
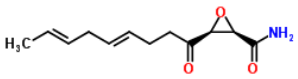
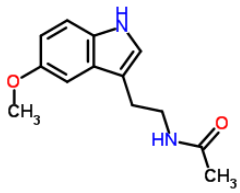
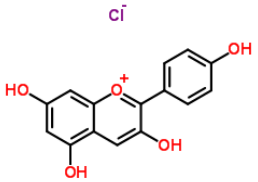
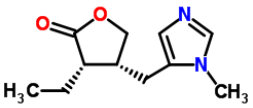
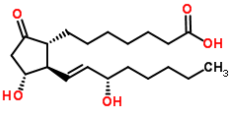
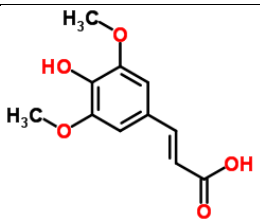
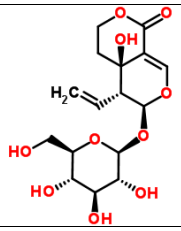
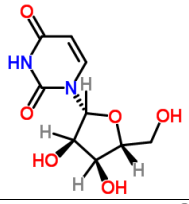
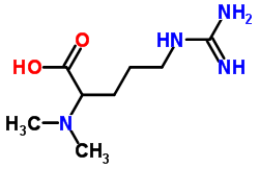
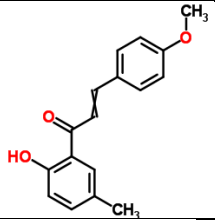
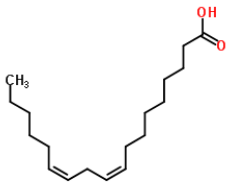
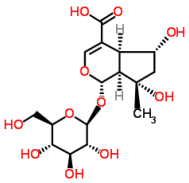
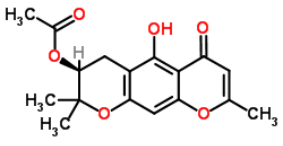
S. No.	Name of components, Formula, Molecular weight (g/mol) and Mass detected	Role in metabolic functions	Source
1.	(2E)-3-(4-Methoxyphenyl)-2-propenyl 6-O-a-L-arabinopyranosyl-b-D-glucopyranoside, C21 H30 O11, (458.1787)	Nephroprotective	**(19)**
2.	[6]-Gingerol, C17 H26 O4 (294.1828)	Anti-tumorigenic	**(20)**
3.	D-erythro-Dihydrosphingosine C18 H39 N O2 (301.2988)	Ceramide in cathepsin D activation	**(21)**
4.	Methyl gallate, C8 H8 O5 (184.0356)	Antioxidant, anticancer and anti-inflammatory	**(22)**
5.	Resveratrol, C14 H12 O3 (228.0788)	Antioxidant	**(23)**
6.	Rutaecarpine, C18 H13 N3 O (287.1061)	Cardio protective	**(24)**
7.	Syringic acid hexoside, C15 H20 O10 (360.1037)	Anti-diabetic	**(25)**
8.	Tyramine, C8 H11 N O (137.0842)	Blood pressure regulator	**(26)**
9.	Asperuloside, C18 H22 O11 (414.1141)	Anti-obesity	**(27)**
10.	Ginkgolide A, C20 H24 O9 (408.1414)	Anti-cerebrovascular disease	**(28)**
11.	Cerulenin C12 H17 N O3 (223.1208)	Antibiotic	**(29)**
12.	Melatonin, C13 H16 N2 O2 (232.1211)	Hormones regulator	**(30)**
13.	Pelargonidin chloride, C15 H11 O5 (271.0598)	Antioxidant	**(31)**
14.	Pilocarpine, C11 H16 N2 O2 (208.1224)	Homeostasis	**(32)**
15.	Prostaglandin E1, C20 H34 O5 (354.2382)	Cardio protector	**(33)**
16.	Sinapic acid, C11 H12 O5 (224.0679)	Antioxidant, anti-inflammatory, anticancer, antimutagenic, antiglycemic, neuroprotective, and antibacterial	**(34)**
17.	Swertiamarin, C16 H22 O10 (374.1217)	Cardiac and metabolic regulator	**(35)**
18.	Uridine, C9 H12 N2 O6 (244.0712)	Neuro regulator	**(36)**
19.	Dimethylarginine, C8 H18 N4 O2 (202.1422)	Atherosclerosis	**(37)**
20.	2'-Hydroxy-5'-methyl-4-methoxychalcone, C17 H16 O3 (268.3071)	Anti-inflammatory	**(38)**
21.	Linolenic acid , C18 H32 O2 (268.1089)	Cardio protective	**(39)**
22.	Shanzhiside, C16 H24 O11 (392.1321)	Regulates angiogenesis	**(40)**
23.	3'-O-Acetylhamaudol, C17 H18 O6 (318.1083)	Anti-tumor	**(41)**
24.	Abscisic acid, C15 H20 O4 (264.1343)	Chromosome regulator	**(42)**
25.	Calciferol, C28 H44 O (396.3394)	Vitamin D activator	**(43)**
26.	Oleuryopein-aglycone, C19 H22 O7 (362.1348)	Neuro vitalizer	**(44)**
27.	Proline-betaxanthin, C14 H16 N2 O6 (308.1014)	Detoxifier	**(45)**
28.	Thymol, C10 H14 O	Broad spectrum antibiotics	**(46)**

	(150.1039)		
29.	(-)-Riboflavin, C17 H20 N4 O6 (376.1375)	Vitamin B2 activator	**(47)**
30.	Sweroside, C16 H22 O9 (358.125)	Hepatoprotective	**(48)**

Fig. 1: Horse gram (*Macrotyloma uniflorum*) cultivar (specific character is dark black and high in protein) collected from high elevation of Almora of Uttarakhand (Table 1)

Fig. 2: Chemical structures of selected constituents (constructed with the help of online web tool www.chemspider.com)

Name of components	**Structure (2D)**	**Name of components**	**Structure (2D)**
"(2E)-3-(4-Methoxyphenyl)-2-propenyl 6-O-a-L-arabinopyranosyl-b-D-glucopyranoside" (C21 H30 O11)		[6]-Gingerol (C17 H26 O4)	
D-erythro-Dihydrosphingosine C18 H39 N O2		Sweroside (C16 H22 O9)	
Methyl gallate (C8 H8 O5)		Resveratrol (C14 H12 O3)	

Rutaecarpine (C18 H13 N3 O)		Syringic acid hexoside (C15 H20 O10)	
Tyramine (C8 H11 N O)		Asperuloside (C18 H22 O11)	
Ginkgolide A (C20 H24 O9)		Cerulenin C12 H17 N O3 223.1208	
Melatonin (C13 H16 N2 O2)		Pelargonidin chloride (C15 H11 O5)	
Pilocarpine (C11 H16 N2 O2)		Prostaglandin E1 (C20 H34 O5)	
Sinapic acid (C11 H12 O5)		Swertiamarin (C16 H22 O10)	
Uridine (C9 H12 N2 O6)		Dimethylarginine (C8 H18 N4 O2)	
2'-Hydroxy-5'-methyl-4-methoxychalcone (C17 H16 O3)		Linolenic acid (C18 H32 O2)	
Shanzhiside (C16 H24 O11)		3'-O-Acetylhamaudol (C17 H18 O6)	

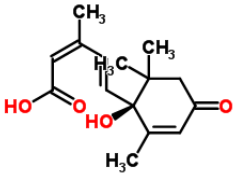
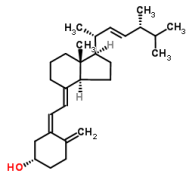
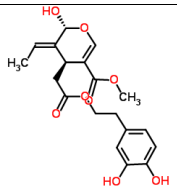
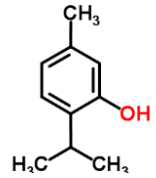
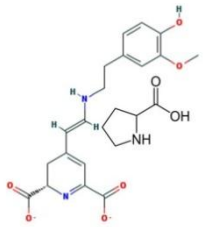
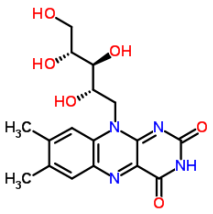
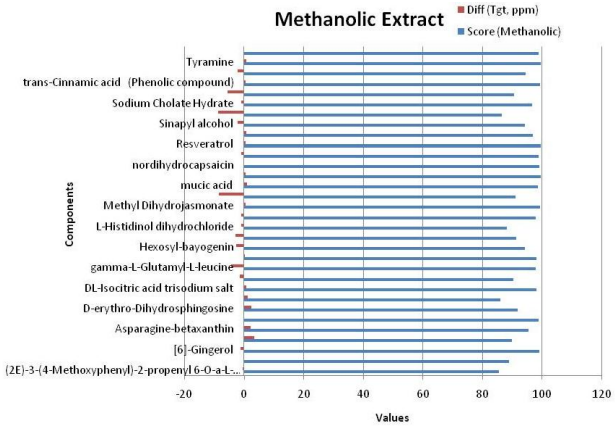
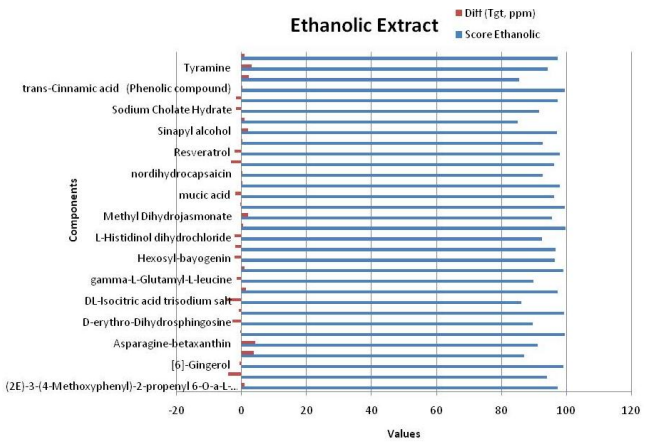
Abscisic acid (C15 H20 O4)		Calciferol (C28 H44 O)	
Oleuropein-aglycone (C19 H22 O7)		Thymol (C10 H14 O)	
Proline-betaxanthin (C14 H16 N2 O6)		(-)-Riboflavin (C17 H20 N4 O6)	

Fig. 3: Comparative analysis of common compound in three extracts with respect of Score and Flag Severity (Diff (Tgt, ppm)) after LC-MS, graph constructed with the help of Microsoft excel. The values of score in between ranged 90-100 and ethanolic extracts have exceeded values of Diff (Tgt, ppm) in compare others (Full data not shown)





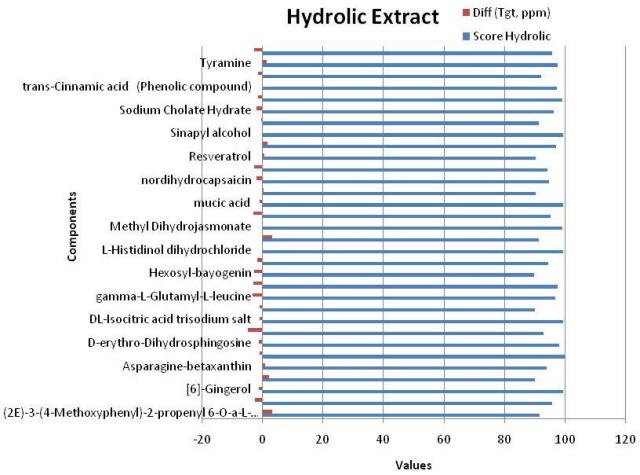
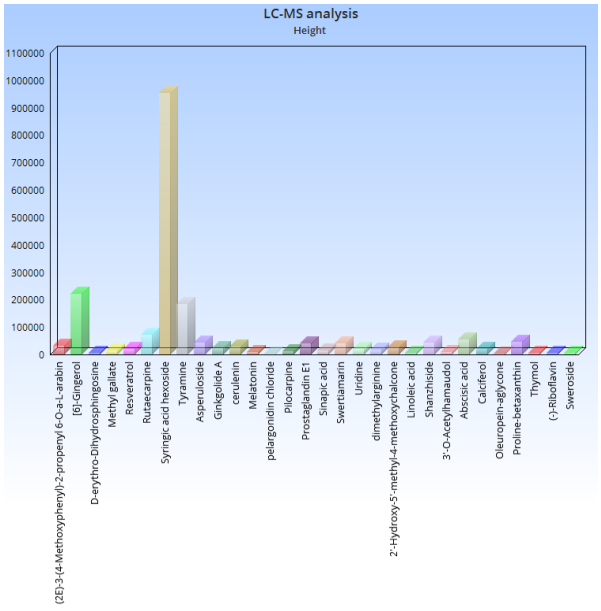
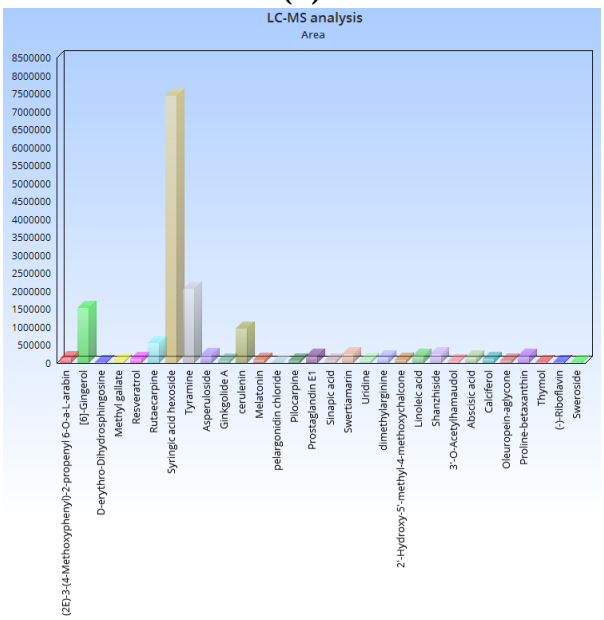


Fig. 4: Bar diagram for height (A) and area (B) of specific peak calculated after LC-MS analysis of selected components. (Bar diagrams were constructed with the help of online tool www.chartgo.com)

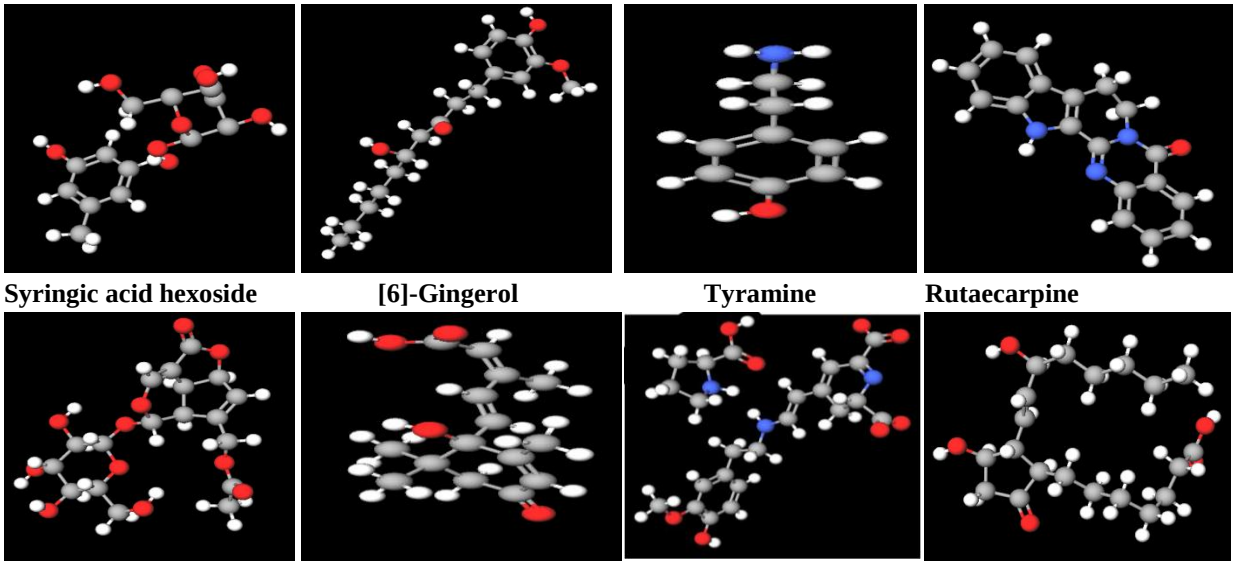


(A)



(B)

Fig. 5: 3D structures of significant compounds found after LC-MS analysis. (Structures constructed with the help of online web www. http://molview.org)



Syringic acid hexoside **[6]-Gingerol** **Tyramine** **Rutaecarpine**

Asperuloside **Abscisic acid** **Proline betaxanthin** **Prostaglandin E1**

CONCLUSION

The present study concludes that the *M. uniflorum* is of varied use and importance in nutrition and therapeutics, the finding of LC-MS analysis unzipped lot of information on the therapeutic compounds present in this less studied seed. The outcome of this investigation could be useful to understand the molecular mechanism of various compounds and nutraceutical value of *M. uniflorum*. Extracts of *M. uniflorum* demonstrated a broad spectrum of nutraceutical efficiency against many human metabolic disorders. To construct or design drugs against many human diseases or metabolic disorders this investigation along with the *In-silico* studies would be of significant use. This is first hand report with complete analysis by LC-MS of *M. uniflorum*, deciphering the HG research in respect to nutraceuticals from high altitude of this Himalayan region.

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