International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 4 | OCT-DEC | 2018 | 416-425
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

PLANT-DERIVED FOOD PRODUCTS AS DIETARY SUPPLEMENTS FOR SUSTAINABLE UTILIZATION- AN *IN VITRO* AND *IN SILICO* APPROACH

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ABSTRACT

Sustainable utilization is the use of components of biological diversity in such a way that does not lead to its long-term decline, thereby maintaining its potential to meet the needs and aspirations of present and future generations. Investigation studies related to dietary supplements from natural edible food products have been one of the significant research interests in recent years. Using several in vitro and in silico methodologies, a study was carried out in leguminous sprouts to analyze the nutrients for its recommendation as dietary supplements for all age groups. The study was carried out with two different samples- horse gram sprouts (Macrotyloma uniflorum (Lam.) Verdc.) and mixed sprouts of combination (Cicer arietinum L. (Chick pea), Macrotyloma uniflorum (Lam.) Verdc. (horse gram) and Vigna radiata (L.) R. Wilczek (Green gram). Screening the phytoconstituents by qualitative phytochemical tests and quantification of the primary and secondary constituents were carried out in methanol and aqueous extracts of fresh and dried sprouts. Phytochemical characterization was carried out through FTIR and GC-MS analysis which proved the presence of essential phytoconstituents like terpenoids, fatty acids, proteins, carbohydrates and vitamins. Antibacterial activity of both the samples were studied against human pathogens namely Staphylococcus aureus, Escherichia coli, Salmonella typhi, Klebsiella pneumoniae and Shigella flexneri. Maximum zone of inhibition were shown by Shigella flexneri in horse gram and in mixed sprouts. Further, bioactive compounds obtained from GC-MS analysis were screened for anti-inflammatory activity and the potent bioactive compound- its structure and functions were analyzed through in silico studies using different bioinformatics tools and softwares which indicated the effective physico-chemical properties of the bioactive compounds which might be responsible for the anti-inflammatory (anti-ulcer) property. Thus, the horse gram sprouts and mixed sprouts enriched with the phytoconstituents can be recommended as a natural source of dietary supplements which can be one of the most economical and easily available source for consumption leading to sustainable utilization.

KEY WORDS

Horse gram sprouts, mixed sprouts, FTIR, GC-MS, antibacterial, in silico studies.

INTRODUCTION

The concept of human development incorporates both economic and social progress and it is recognized that the use of resources are involved in both of these aspects. Sustainable utilization is the use of components of biological diversity in such a way that it does not lead to its long-term decline, thereby maintaining its potential to meet the needs and aspirations of present

and future generations. There is a need for nutritionally balanced, energy- dense, easily digestible foods with functional benefits to be formulated in low cost [1].

Sprouts are formed from seeds during sprouting. The sprouts are known to be an excellent source of proteins, vitamins and minerals. Sprouts when consumed at its growing phase, the nutrient concentration remains very high. Besides the essential nutrients, the sprouts contain enormous phytochemicals. The phytoconstituents, vitamins, minerals, enzymes and amino acids are of great importance as these are the most useful in maintaining human health [2]. The bioactive compounds have a broad spectrum of anti-bacterial, anti-fungal, antioxidant and anti-inflammatory properties [3]. Legumes are one of the most important sources of food supplies, especially in developing countries in terms of food energy as well as nutrients.

Since ancient times, mung bean and soybean sprouts have been grown in Asia, have now found their way into Western cuisine because of the presence of several phytoconstituents. Generally, pulses, cereals and oil seed crops are used for sprouting but apart from these, several vegetables and herbs are used for sprouting which includes, mustard, peas, cabbage, radish, spring onion, broccoli etc. Several sprouts or different varieties of the same sprouts can be mixed to create new combinations of nutrients, textures, flavours and colours [4].

The leguminous sprout samples used in the present study. Sample I was horse gram sprouts and sample II was mixed sprouts. The main objective of the study was to do qualitative and quantitative analysis of the phytoconstituents, characterization of the phytochemicals by FTIR and GC-MS analysis, performing bioassays for antibacterial, anti-inflammatory and antioxidant activity of the sprouts and *in silico* analysis of physico-chemical properties of the bioactive compounds followed by docking studies for showing the functional applications of the confirmed compounds in the sprouts.

MATERIALS AND METHODS

Collection of samples and sprouting

Horse gram seeds (*Macrotyloma uniflorum* (Lam.) Verdc.), chick pea seeds (*Cicer arietinum* L.) and green gram seeds (*Vigna radiata* (L.) R. Wilczek) were purchased from horticultural society, Chennai, Tamil Nadu, India. Sample I was horse gram sprouts and sample II was mixed sprouts (chick pea, horse gram and green gram- 100g each). The experimental seeds were germinated, these fresh sprouts were used for further studies. The analysis of dried samples was carried out with 200gms of each of the samples using shade dry method for three weeks and the sprouts were ground using a blender and stored in air tight containers for further analysis.

Preparation of crude extracts, Qualitative phytochemical screening and Quantification of the phytoconstituents

The crude extract preparation of the fresh and dried form of both the samples were carried out using solvents like butanol, acetone, methanol and water (aqueous) separately (1:10) using cold percolation method [5]. Qualitative phytochemical tests for the identification of the primary and secondary phytoconstituents in the butanol, acetone, methanol and aqueous extracts (HB, HAc, HM, HA, HBD, HAcD, HMD, HAD and MB, MAc, MM, MA, MBD, MAcD, MMD and MAD) in fresh and dried horse gram sprouts and mixed sprouts were carried out using standard protocols [6, 7]. The methanol and aqueous extracts of the fresh and dried samples which showed good results were taken for further analysis. Using UV Spectrophotometer (UV 1650PC Shimadzu), the quantification of the phytoconstituents such as total soluble sugars [8, 9], proteins [10], flavonoids [11], terpenoids [12] were carried out and the amount of phytic acid was also quantified [13].

Fourier Transform Infrared Spectrophotometer (FTIR) analysis

The methanol and aqueous extracts of the fresh and dried sprout samples were prepared. The extracts were evaporated by flash evaporator, which was then mixed with KBr salt, using a mortar and pestle and compressed into a thin pellet. Infrared spectra were recorded on KBr pellet on a Shimadzu FTIR spectrometer 4000 – 500cm^{-1}.

Antibacterial assay, *in vitro* anti-inflammatory and anti-oxidant assays

Different concentrations of the extracts of the samples (50µg/ml, 75µg/ml, 100µg/ml) was assayed against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumonia*e and *Shigella flexneri* (Bacterial cultures obtained from Department of Microbiology, Ethiraj College for Women, Chennai)

were used. Antibacterial assay was carried out by well diffusion method using Mueller-Hinton agar media. Streptomycin was used as positive control. Triplicates were maintained for all the samples. Zone of inhibition around the well was observed after 24 hours.

In vitro anti-inflammatory assay was carried out using the method of inhibition of the albumin denaturation using UV Spectrophotometer (UV 1650PC Shimadzu) [14]. *In vitro* antioxidant assay, 1, 1- diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity [15] of the methanol and aqueous extracts of the fresh and dried horse gram sprouts were analysed through standard methods (using UV Spectrophotometer- UV 1650PC Shimadzu). The experiment was conducted in triplicates and values were expressed as equivalents of ascorbic acid in µg/mg of the extract.

Gas Chromatography Mass Spectrometry (GC-MS) analysis and *in silico* studies

GC-MS analysis of the fresh horse gram sprouts methanol extract was carried out by the standard method [16]. The compounds identified in GC-MS studies were screened against the target protein (*Helicobacter pylori*) to study the anti-ulcer property. Using several bioinformatic tools and softwares, the physico-chemical properties of the bioactive compounds were studied. Then the target protein molecule was retrieved from (PDB) Protein Data Bank. By using standard protocol [17], docking was carried out to prove the anti-inflammatory property of the bioconstituents from the sprout. Diclofenac sodium was used as a standard.

Statistical analysis

For each experiment, data presented are the means of three replicates. Values are expressed as mean ±SD of three replicates.

RESULTS AND DISCUSSION

Qualitative phytochemical screening

The fresh and dried horse gram and mixed sprouts revealed the presence of alkaloids, saponins, terpenoids, glycosides, steroids, triterpenoids, resin, quinone, proteins, amino acids, carbohydrates, flavonoids, cardiac glycosides, tannins, phenols, fixed oils, fats and fatty acids. Among the samples analysed for phytoconstituents in four different solvents, methanol and aqueous solvents showed prominent results both in fresh and dry samples. Hence further work was carried out only in methanol and aqueous extracts. The chemical substances in the plants or in crude extracts are said to be biologically active metabolites. These might be primary or secondary metabolites. The secondary metabolites are mainly involved in the significant pharmacological activities like antimicrobial, anti-inflammatory, antioxidant activities. In comparison, the horse gram sprouts and mixed sprouts contain equal amount of phytoconstituents which are mainly responsible for biological significance of being as dietary supplements for sustainable utilization.

Quantification of the phytoconstituents

The methanol extract of both the fresh sprout samples showed maximum total soluble sugars content (0.58 ± 0.2mg/g of glucose and 0.57 ± 0.2mg/g of glucose) followed by methanol extract of both the fresh sprout samples showed maximum protein content (37 ± 0.9mg/ml of protein and 38 ± 0.6mg/ml of protein). Then the methanol extract of both the fresh sprout samples showed maximum flavonoid content (0.26 ± 0.11mg QE/g and 0.25 ± 0.009mg QE/g) followed by methanol extract of both the fresh sprout samples showed maximum terpenoid content (88 ± 1.4mg/g and 89 ± 1.3mg/g of terpenoid). The phytic acid content in the fresh and dried sprouts was found to be less when compared to the control seeds used for the samples where horse gram seeds had 1.4 ± 0.3mg/g and mixed sprouts seeds had 1.5 ± 0.2mg/g of phytic acid.

In the present study, the quantitative analysis revealed that methanol extract of fresh horse gram sprouts had maximum total soluble sugars, proteins, flavonoids, terpenoids with less amount of phytic acid. These several compounds have broad biological and pharmacological activities. Legumes contain a major anti-nutritional factor (phytic acid) which prevent protein digestibility in our body. The phytase enzyme, an acid phosphatase, hydrolyze phytic acid to a series of lower phosphate esters of myoinositol and phosphate. The results were promising with the decreased levels of phytic acid content when compared to the seeds.

Fourier Transform Infrared Spectrophotometer (FTIR) analysis

FTIR spectra of the methanol and aqueous extracts of fresh horse gram sprouts showed the presence of alkyl halides, aromatics, esters, alkanes, amides, alkenes, phosphines and alcohols whereas the functional group, alkynes are restricted only to methanol extract and aromatic compounds, thiols are found only in aqueous

extract. FTIR spectra of the methanol and aqueous extracts of dried horse gram sprouts showed the presence of alkyl halides, alkenes, esters, amides, nitro compounds, alkanes and carboxylic acids whereas silane compounds are found only to methanol extract and alkynes are restricted to aqueous extract.

Various functional groups such as alkyl halides, alkenes, aromatics, esters, amides, nitro compounds, phosphines, alkanes and alcohols were found in both methanol and aqueous extracts of fresh mixed sprouts whereas silane compounds was restricted only to fresh mixed sprouts. Several functional groups like alkyl halides, alkenes, esters, phosphines, alkanes and alcohols were observed both in methanol and aqueous extracts of dried mixed sprouts but aldehydes was restricted to methanol extract of dried mixed sprouts and alkynes was restricted to aqueous extract of dried mixed sprouts.

The results revealed terpenoids were found in significant amount in fresh and dried horse gram sprouts because of the presence of C-H stretch at 2928.07 cm^{-1} in methanol extract and 2927.1 cm^{-1} in aqueous extract of fresh horse gram sprouts. The dried horse gram sprouts methanol extract had terpenoids with C-H stretch at 2930 cm^{-1} and in aqueous extract at 2928.07 cm^{-1}. In fresh and dried mixed sprouts, terpenoids were found in significant amount due to the presence of C-H stretch at 2928.9 cm^{-1} in methanol extract and 2924.21 cm^{-1} in aqueous extract of fresh mixed sprouts. The dried mixed sprouts had terpenoids with C-H stretch at 2926.14 cm^{-1}. The presence of varied functional groups may be attributed to the existence of variety of potential phytoconstituents. In comparison, mixed sprouts had a variety of functional groups than horse gram sprouts. However, terpenoids were found in higher amounts in both the sprout samples.

Antibacterial assay

Methanol extract of fresh horse gram sprouts (HM) at 100µg showed maximum zone of inhibition of (30 ± 0.4mm) (Figure 1) and methanol extract of fresh mixed sprouts (MM) showed maximum zone of inhibition (30 ± 0.6mm) against *Shigella flexneri* (Figure 2).

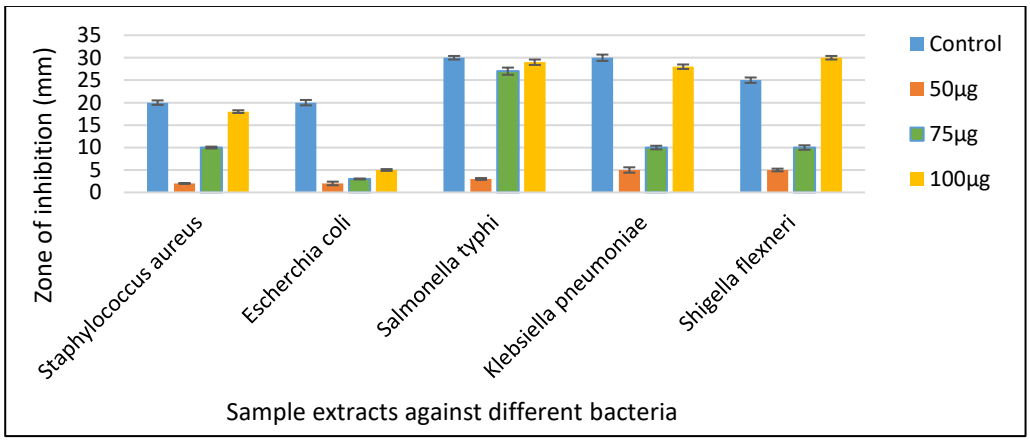

Figure 1: Antibacterial activity of methanol extract of fresh horse gram sprouts against food borne bacteria

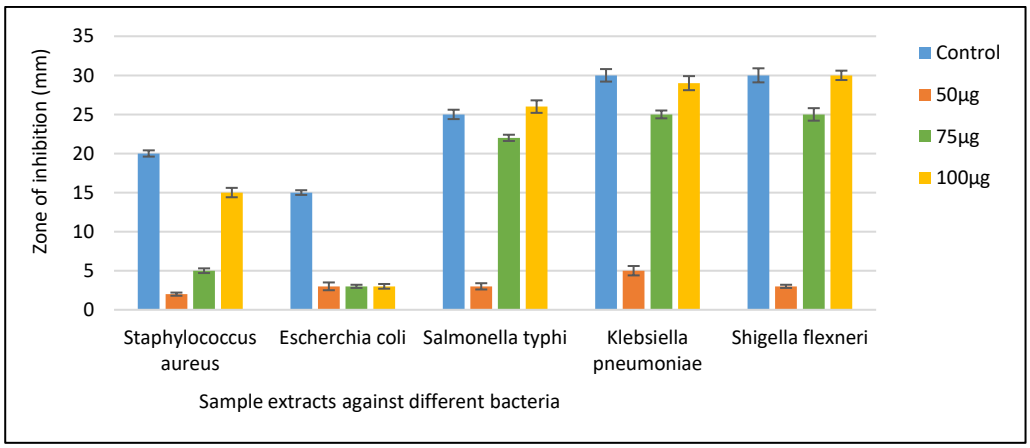

Figure 2: Antibacterial activity of methanol extract of fresh mixed sprouts against food borne bacteria

The increasing antibiotic resistance of some pathogens that are mainly associated with food borne illness is one of the major concerns. Thus, there is growing interest in using natural antibacterial compounds. The presence of potent phytoconstituents like terpenoids and flavonoids in both horse gram and mixed sprouts might be responsible for the prominent antibacterial activity of the sprouts. In comparison, even though horse gram sprouts showed potent activity, mixed sprouts also showed significant antibacterial activity. Thus, the sprout samples act as a natural antibacterial agent.

***In vitro* anti-inflammatory assay**

The anti-inflammatory assay carried out at different concentrations- 100, 200, 300, 400, 500µg of methanol and aqueous extracts of both the sprout samples revealed inhibition of thermally induced protein denaturation in dose dependent manner. However, the methanol extracts of fresh sprout samples (HM and MM) showed per cent maximum inhibition of 83 ± 1.1 and 81 ± 1.3 at 500µg concentration with IC_{50} value of 225.3µg/ml and 239.3µg/ml (Figure 3 & 4). The anti-inflammatory activity of standard diclofenac sodium showed per cent maximum inhibition 90 ± 1.5 at 500µg concentration with IC_{50} value of 125.8µg/ml.

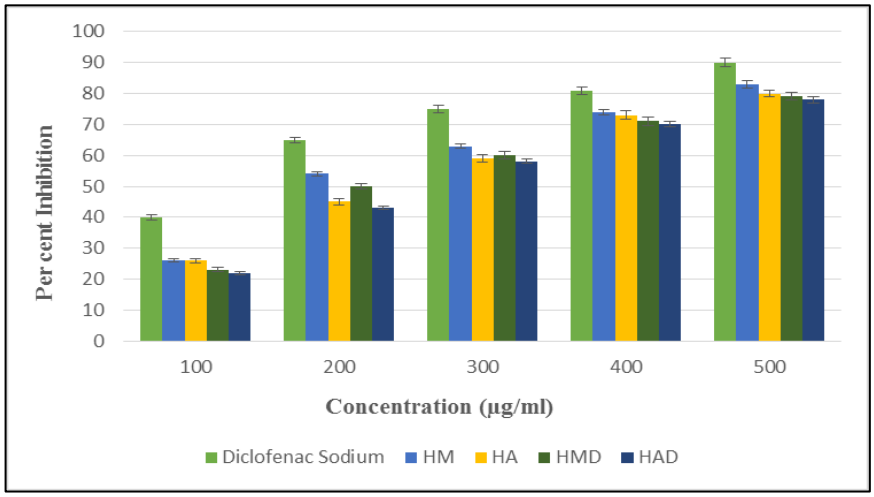

Figure 3: Anti-inflammatory activity of fresh and dried horse gram sprouts

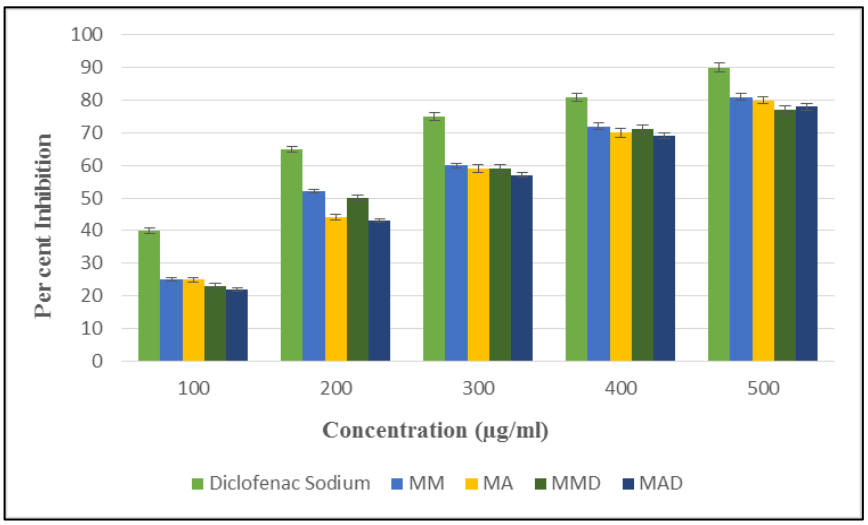

Figure 4: Anti-inflammatory activity of fresh and dried mixed sprouts

The main cause of inflammation is protein denaturation. Agents that can prevent denaturation of proteins would be significant and are called as anti-inflammatory agents. In the present study, the methanol extract of fresh sprouts samples showed maximum anti-inflammatory activity than the dried sprouts. The results

obtained are the clear evidence for consumption of the horse gram sprouts and mixed sprouts which could be a potent anti-inflammatory agent due to the presence of essential phytoconstituents such as terpenoids and flavonoids which has a potent biological effect of stabilization of lysosomal membranes.

***In vitro* anti-oxidant assay**

In vitro anti-oxidant assay using DPPH carried out at 100, 200, 300, 400, 500µg concentrations of methanol and aqueous extracts of both the sprout samples revealed decolourization property in dose dependent manner. However, the methanol extracts of fresh sprout samples (HM and MM) showed per cent maximum inhibition of 86 ± 1.3 and 85 ± 1.1 at 500µg concentration with IC_{50} value of 180.9µg/ml and 187.8µg/ml (Figure 5 & 6). The DPPH scavenging activity of standard ascorbic acid showed per cent maximum inhibition of 90 ± 1.5 at 500µg concentration with IC_{50} value of 149.1µg/ml.

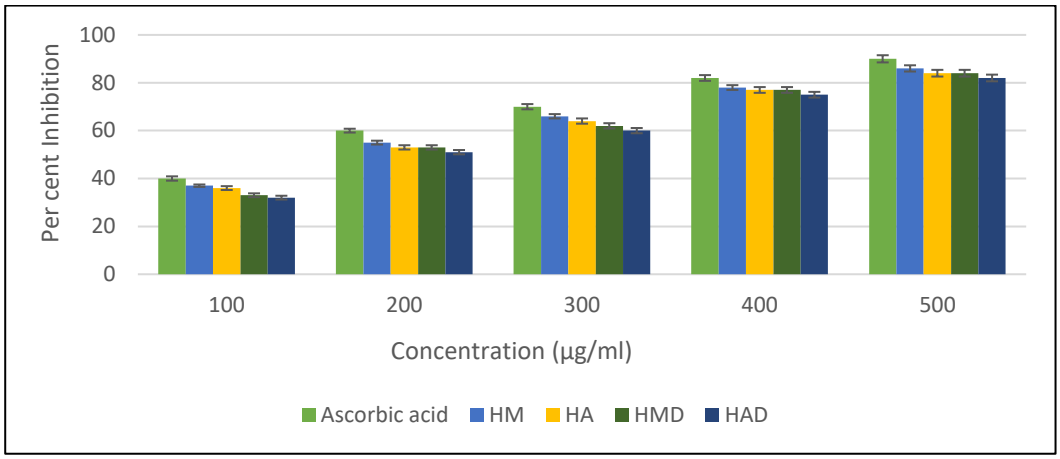

Figure 5: DPPH assay of fresh and dried horse gram sprouts

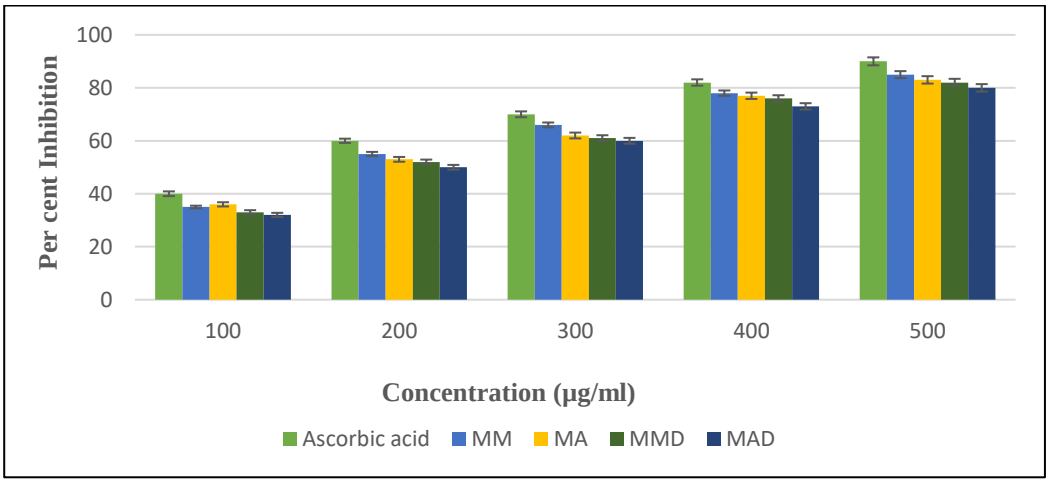

Figure 6: DPPH assay of fresh and dried mixed sprouts

In natural products, the electron donation ability can be determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) purple- coloured solution bleaching where the method is based on scavenging of DPPH through the addition of a radical antioxidant which results in the decolourization of the DPPH solution. The degree of the colour change is proportional to the concentration and potency of the antioxidants. In comparison, mixed sprouts had antioxidant activity on par with the horse gram sprouts. Thus, this combination of mixed sprouts can be a potent antioxidant enriched with phytoconstituents.

Gas Chromatography Mass Spectrometry (GC-MS) analysis

GC-MS is one of the techniques to identify the bioactive constituents of long chain branched chain hydrocarbons, alcohols, acids, esters, etc. The bioactive compounds in the extract were identified using NIST database on comparison with actual mass spectral obtained. The GC-MS spectrum of methanol extract of

fresh horse gram sprouts (HM), indicated the presence of various compounds like diglycerol, DL-Proline, DL-Phenylalanine, 1,3-Propanediol, quinolone, vinyl caprylate, Beta-D-Mannofuranoside, myo-inositol, isopropyl myristate, oxirane, ascorbic acid, n-nonadecanol-1, cis-vaccenic acid, 1-hepatocosanol, gamma-linolenic acid, methyl ester, glycerol tricaprylate, stigmasterol, geranyl geraniol, gamma-sitosterol, fumaric acid and gamma tocopherol (Figure 7). The GC-MS spectrum of methanol extract of fresh mixed sprouts (MM), indicated the presence of bioactive compounds like dodecanoic acid, alpha-D-glucopyranoside, myo-inositol, vinyl caprylate, methyl mannose, cis-vaccenic acid, geranyl geraniol, gamma sitosterol and gamma tocopherol (Figure 8).

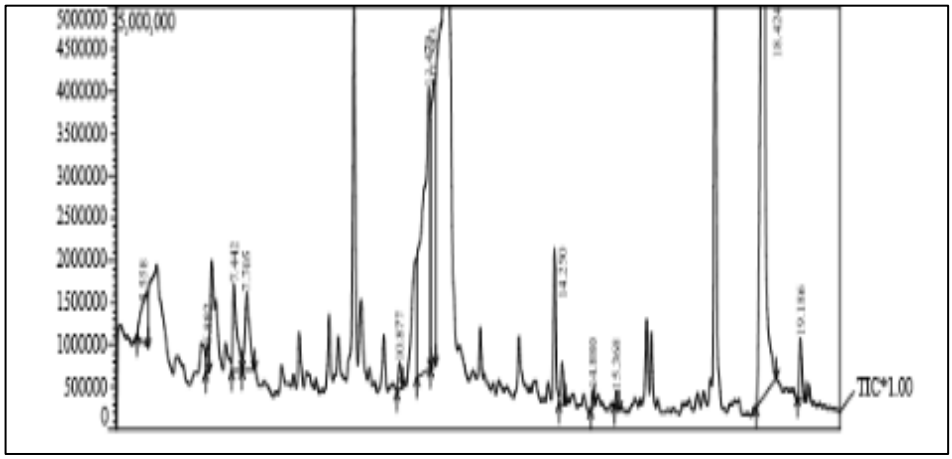

Figure 7: GC-MS spectrum of methanol extract of fresh horse gram sprouts

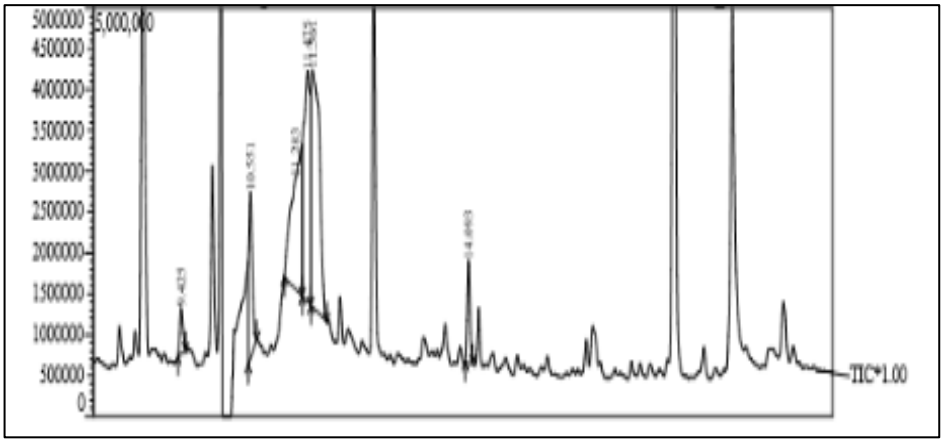

Figure 8: GC-MS spectrum of methanol extract of fresh mixed sprouts

The compounds are mostly of terpenoids, fatty acids, carbohydrates, amino acids and other small functional groups. These compounds are mainly involved in several metabolic pathways thereby resulting in antibacterial, anti-inflammatory and antioxidant properties. Thus, the present study strongly supports the significant antibacterial, anti-inflammatory and antioxidant activities which may be due to the presence of a wider range of bioconstituents reported. Thus, the GC-MS studies are further taken for *in silico* analysis with the therapeutic compounds identified for several biological activities especially with anti-inflammatory and antiulcer properties.

***In silico* analysis of physico-chemical properties and docking studies**

From the GC-MS analysis, screening was carried out for the compounds related to anti-ulcer property. DL-Proline, a free amino acid produced from methanol extract of fresh horse gram sprouts and Geranyl geraniol, a diterpene from fresh mixed sprouts methanol extract (MM) tend to have anti-ulcer property. By using the tool, Prot Param, several physico-chemical properties such as the number of amino acids, molecular weight, theoretical (pI) value, extinction coefficients, instability index, aliphatic index and grand

average of hydropathicity of the bioactive compounds were analysed (Table 1).

Table 1: Physico-chemical properties of DL-Proline and Geranyl geraniol from fresh horse gram and mixed sprouts (Methanol extracts)

Physico-chemical properties	DL-Proline	Geranyl geraniol
Number of amino acids	112	115
Molecular Weight	12886.7	13654.6
Theoretical (pI) Value	5.60	5.75
Extinction coefficients (EC)	8605M^{-1} cm^{-1}	8605M^{-1} cm^{-1}
Instability Index (II)	21.18	22.16
Aliphatic Index (AI)	70.78	71.90
Grand Average of Hydropathicity (Gravy)	0.350	0.370

Extinction coefficient of DL-Proline and Geranyl geraniol at 280 nm was 8605 $M^{-1}cm^{-1}$. The extinction coefficient (EC) clearly indicates the amount of light absorption of proteins at a particular wavelength. It indicates the presence of Cys, Trp and Tyr. These amino acids (Trp, Tyr, Cys) are considered to be an important parameter in the calculation of extinction coefficient of proteins [18].

Instability index is mainly used to determine whether the compounds will be stable for longer time. If the index is less than 40, it is probably stable. If the value is greater than 40, it is probably not stable. The instability index of DL-Proline and Geranyl geraniol was shown to be 21.18 and 22.16 and this shows that these are stable. The instability index value for the dicer proteins were found to be ranging from 21.90 to 47.14. The results imply XP_003553805 as stable protein [19].

The aliphatic index of DL-Proline and Geranyl geraniol was found to be 70.78 and 71.90 indicated that the protein may be stable for a wide range of temperatures. The aliphatic index is a measure of the relative volume occupied by aliphatic side chain of the amino acids such as alanine, valine, leucine and isoleucine [20].

GRAVY value is the sum of hydropathy values of all the amino acids. A hydropathy scale which is based on the hydrophobic and hydrophilic properties of the 20 amino acids is used. GRAVY values of DL-Proline and Geranyl geraniol was 0.050 to 0.370. The very low GRAVY index clearly indicated that the protein could better interact with water. The very low GRAVY index of DCLs EEE81952, XP_003553805 and XP_002268369 implies that these DCLs could result in a better interaction with water [20].

The present study clearly indicates that anti-ulcer compounds like DL-Proline (free amino acid) from horse gram sprouts and Geranyl geraniol (diterpene) from mixed sprouts possesses strong physico-chemical properties. These physico-chemical properties of the phytoconstituents might be responsible for the sprouts being a natural dietary supplement.

It is reported that peptic ulcer is caused by *Helicobacter pylori,* a gram-negative bacillus or non-steroidal anti-inflammatory drugs found in the digestive tract in the stomach or the duodenum [21]. The pathogenic activity is found in the Type-I strains of the bacteria which encodes the effector protein cytotoxin-associated gene (cagA) [22]. The target protein of *Helicobacter pylori* was obtained from Protein Data Bank (http://www.rcsb.org/pdb/)- PDB ID: 1G60. Diclofenac sodium, a non-steroidal anti-inflammatory agent primarily available as the sodium salt was used as standard. More negative values are indication of higher binding affinity which indicated the strong anti-ulcer property. Docking analysis carried out using Mcule database, DL-Proline from fresh horse gram sprouts methanol extract (HM) showed docking scores of -4.7, -4.5, -4.2 and -4.1 (Figure 9). Docking analysis of geranyl geraniol from fresh mixed sprouts methanol extract (MM) showed docking scores of -6.9, -6.6, -5.9 and -5.8 (Figure 10). Docking analysis of standard diclofenac sodium showed docking scores of -7.5, -7.3, -7.1 and -6.8 (Figure 11).

Horse gram sprouts and mixed sprouts showed prominent binding affinity against *Helicobacter pylori* on par with the standard drug compound diclofenac sodium. The docking results are a clear evidence for

horse gram and mixed sprouts having anti-ulcer properties. Thus, the horse gram sprouts and mixed sprouts with enriched therapeutic phytoconstituents can be recommended as a natural edible product for ulcer problems.

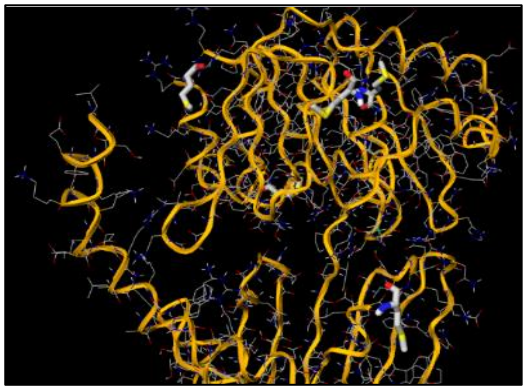

Figure 9: DL-Proline docked with *Helicobacter pylori*

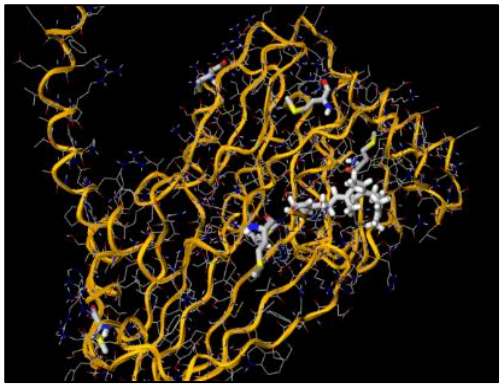

Figure 10: Geranyl geraniol docked with *Helicobacter pylori*

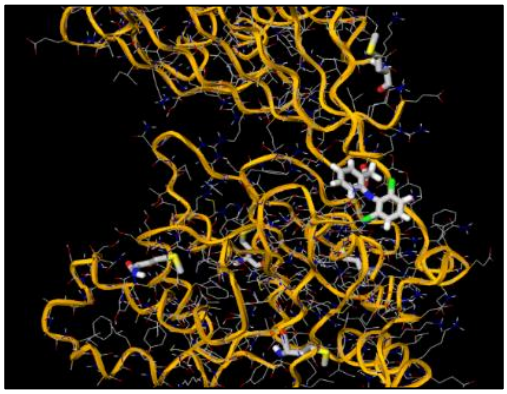

Figure 11: Standard Diclofenac sodium docked with *Helicobacter pylori*

CONCLUSION

Based on the bioassays and *in silico* analysis, the horse gram sprouts (HM) and mixed sprouts (MM) with phytoconstituents such as proteins, carbohydrates, terpenoids and flavonoids possess strong antibacterial, anti-inflammatory and anti-oxidant activities. GC-MS and *in silico* studies confirmed the presence of lead compounds, DL-Proline, a free amino acid from fresh horse gram sprouts and Geranyl geraniol, a diterpene from fresh mixed sprouts (indicating the effective physico-chemical properties of the bioactive compounds which might be responsible for the anti-inflammatory (anti-ulcer) property). Thus, the horse gram sprouts and mixed sprouts enriched with the phytoconstituents can be recommended as a natural source of dietary supplements which can be one of the most economical and easily available sources for consumption leading to sustainable utilization.

Acknowledgement

The authors thank Mrs. Prema Sampathkumar, Former Associate Professor and Head, Mrs. V. Manimozhi, Associate Professor and Head, the Faculty members and supporting staff of Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women (Autonomous), Chennai- 600 008 and Dr. A. Nirmala, Former Principal and Dr. Mrs. S. Bhuvaneswari, Principal i/c, Ethiraj College for Women (Autonomous) for their valuable support, encouragement throughout the entire period of research. We would also like to express our sincere thanks for the facilities extended by the Central Instrumentation Centre of Ethiraj College for Women.

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Received:06.08.18, Accepted: 09.09.18, Published:01.10.2018

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