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Characterization of phytopharmaceuticals from fresh and dried sprouts of *Macrotyloma uniflorum* (Lam.) Verdc.

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Abstract : Dietary supplements from different plant sources and plant products are referred to as 'Phytopharmaceuticals'. These are mainly produced from different parts of the plants either in fresh or dried forms. Currently, natural products are well recognized in the pharmaceutical industry for their broad significant pharmacological activities. Bioactive natural products often occur as a part of a family of related molecules which is of great value to isolate a number of homologues and obtain structure-activity information. One such effective natural product from plant source are sprouts which contain essential bioactive components with less anti-nutritional factor especially phytic acid that can lead to the improvement of the food technologies and to healthy nutrition supplements. The present study was carried out to analyse the phytoconstituents present in fresh and dried sprouts of *Macrotyloma uniflorum* (Lam.) Verdc. (Horse gram). Screening the bioconstituents through preliminary qualitative phytochemical tests and quantification of the primary and secondary constituents were carried out in fresh aqueous and methanol extracts. The characterization of the phytoconstituents were analysed through FTIR. Specific bioactive compounds were identified through GC-MS studies. Antibacterial activity of the horse gram sprouts against several human pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumonia*e and *Shigella flexneri* were studied. Maximum zone of inhibition were shown by *Shigella flexneri*, *Salmonella typhi* and *Klebsiella pneumonia*e. *In-vitro* antioxidant and anti-inflammatory studies proved the presence of 'Phytopharmaceuticals' such as terpenoids, proteins, carbohydrates, fatty acids and vitamins. Through *insilico* analysis, docking studies were performed to confirm the functional role of the specific phytopharmaceuticals. Thus, the fresh and dried horse gram sprouts are enriched with the significant phytopharmaceuticals which can be recommended as a good source of natural therapeutic agents.

Keywords : Horse gram sprouts, phytopharmaceuticals, antibacterial, antioxidant, anti-inflammatory, *insilico* analysis.

Introduction

Dietary supplements from different plant sources are referred to as 'Phytopharmaceuticals'. Natural products contain essential phytoconstituents which are capable of curing various diseases by acting as a plant medicine and are the source for drug designing processes. Hence, it is necessary to explore the phytochemicals[1]. Bioactive natural products often occur as a part of a family of related molecules which is of great value to isolate a number of homologues and obtain structure-activity information. Recent researches reveals that many phytoconstituents such as proteins, vitamins, minerals, carbohydrates, terpenoids, tannins, phenols, flavonoids can protect human against diseases for which it is studied extensively to establish their efficacy and to

understand the underlying mechanism of their action. Secondary plant metabolites have biological propertiessuch as antioxidant activity, antimicrobial effect, anti-inflammatory activity, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property[2].

Horse gram, *Macrotylom auniflorum*(Lam.) Verdc.[3]also described as *Dolichos biflorus* L. in the literature[4]. Horse gram sprouts are used in eliminating kidney stones. It also helps in lowering cholesterol levels and could play a role in anti-oxidation[5]. Kaempferol -3-0B-D-glucosid, B-Sitosterol and Stigmasterol were investigated and recently reported the cytotoxicity assessment of the horse gram[6]. It contain essential bioactive components with less anti-nutritional factor especially phytic acid. Phytic acid is present in the seeds gets degraded due to the phytase enzyme produced during sprouting.

Currently, there is a need for nutritionally balanced, energy- dense, easily digestible foods with functional benefits to be formulated in low cost. Horse gram sprouts are easily available and is of great biological value in improving the health aspects. Therefore, the main objective of the present work is to screen the qualitative and quantitative phytoconstituents, to analyse the antibacterial, anti-inflammatory and antioxidant potential of horse gram sprouts using different solvents. FT-IR and GC-MS studies were carried out to characterize the presence of bioactive compounds in the sprout extract. The functional role of selected potent phytoconstituent from GC-MS studies was further investigated by *in-silico* analysis.

Experimental

Sample collection and germination

The fresh sprouts of *Macrotyloma uniflorum*(Lam.) Verdc. (Horse gram) were purchased from horticultural society, Chennai, Tamil Nadu. These fresh sprouts were used for further studies. The analysis of dried samples was carried out with 200gms of the horse gram sprouts using shade dry method for three weeks and the sprouts were ground using a blender and stored in air tight containers for further analysis (Figure 1 and 2).

Figure 1.Fresh horse gram sprouts

Figure 2.Dried horse gram sprouts powder

Screening of microbial contamination

The fresh and dried horse gram sprouts were checked for bacterial and fungal contamination using serial dilution method. Dilutions 10^{-6} and 10^{-7} were used for analysis for bacterial contamination and dilutions 10^{-3} and 10^{-4} were used for analysis for fungal contamination. Nutrient Agar and Potato Dextrose Agar plates were used for the analysis of bacterial and fungal contamination respectively by pour plate method. Nutrient agar plates were incubated at 37°C and PDA plates were kept in room temperature. The plates were observed after 24 hours for any bacterial growth and 48 hours for fungal growth.

Preparation of crude extracts and Qualitative phytochemical screening

The crude extract preparation of the fresh and dried form was carried out using 10gms of the sprouts ground with 100ml of each of the solvents like butanol, acetone, methanol and water (aqueous) separately using cold percolation method[7]. Preliminary qualitative phytochemical tests for the identification of the primary and secondary phytoconstituents in the butanol, acetone, methanol and aqueous extracts (HB, HAc, HM, HA and HBD, HAcD, HMD, HAD) in fresh and dried horse gram sprouts were carried out using standard protocols[8,9].

Quantification of the phytoconstituents

The methanol and aqueous extracts of the fresh and dried horse gram sprouts which showed good results were taken for further study. Using UV Spectrophotometer (UV 1650PC Shimadzu), the quantification of the phytoconstituents such as total soluble sugars[10,11], proteins[12], flavonoids[13], terpenoids[14]were carried out and the amount of phytic acid was also quantified[15].

Characterization by Fourier Transform Infrared Spectrophotometer (FT-IR) analysis

For the FT-IR analysis, Spectrum FT-IR system (Shimadzu, IR Affinity 1, Japan), equipped with a DLATGS detector with a mirror speed of 2.8mm/sec. scan range: from 400-4000cm^{-1} with a resolution of 4cm^{-1} was used. The methanol and aqueous extracts of the fresh and dried horse gram sprouts were prepared. These extracts were evaporated by flash evaporator, which was then mixed with a 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

Different concentrations of the extracts of the samples (50µg, 75µg, 100µg) 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 and Anti-oxidant assays**

In-vitro anti-inflammatory assay was carried out using the method of inhibition of the albumin denaturation using UV Spectrophotometer (UV 1650PC Shimadzu)[16].*In-vitro*antioxidant assays like hydrogen peroxide (H_2O_2) scavenging assay[17], reducing poweractivity[18] and 1, 1- diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity[19] of the methanol and aqueous extracts of the fresh and driedhorse 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 *Insilico* docking studies

GC-MS analysis of the fresh horse gram sprouts methanol extract was carried out by the standard method[20].The compounds identified in GC-MS studies were screened against the target protein (*Helicobacter pylori*) to study the anti-ulcer property. The target molecule was retrieved from (PDB) Protein Data Bank. The details of the bioactive compounds were retrieved from the Pubchem. By using standard protocol[21], 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

Screening of microbial contamination

The fresh and dried horse gram sprouts were examined for microbial contamination to check the purity of the samples used after 24 hours and 48 hours, where after 24 hours, microbial population was found to be nil and following are the results recorded after 48 hours. Results revealed that horse gram sprouts were found to be pure with very minimal microbial contamination (Table 1). Legumes are one of the important food components in vegetarian populations because of their high nutritional factors and these are most probably consumed after processing in different forms. The complete sterilization during germination, drying and powdering of the seeds may be responsible for maintaining the quality of the sample used since these sprouts are consumed either in fresh or dried form.

Table 1. Screening of microbial contamination of fresh mixed sprouts

S.No.	Sample	Bacteria			Fungi		
		Control	10^{-6}	10^{-7}	Control	10^{-3}	10^{-4}
1.	Fresh Horse gram sprouts	-	-	-	-	-	1
2.	Dried Horse gram sprouts	-	-	1	-	1	-

Qualitative phytochemical screening

The phytochemical analysis of fresh and dried horse gram sprouts using solvents namely butanol, acetone, methanol and aqueous revealed the presence of alkaloids, saponins, terpenoids, glycosides, steroids, triterpenoids, resin, quinone, proteins, amino acids, carbohydrates, flavonoids, cardiac glycosides, phenols, fixed oils, fats and fatty acids chiefly in methanol and aqueous solvents. The fresh horse gram sprouts also showed the presence of tannins (Table 2).

Table 2. Phytochemical analysis of solvent extracts of fresh and dried horse gram sprouts

S.No.	Phytochemical Constituents	Butanol		Acetone		Methanol		Aqueous	
		F	D	F	D	F	D	F	D
1.	Alkaloids	-	-	-	-	-	-	+	+
2.	Saponins	+	+	+	+	+	+	+	+
3.	Terpenoids	+	+	+	+	+	+	+	+
4.	Glycosides	-	-	+	+	+	+	+	+
5.	Steroids and Triterpenoids	+	+	+	+	+	+	+	+
6.	Resin	+	+	+	+	+	+	+	+
7.	Quinone	+	-	+	-	+	+	+	+
8.	Gum and Mucilage	-	-	-	-	-	-	-	-
9.	Coumarin	-	-	-	-	-	-	-	-
10.	Anthroquinone	-	-	-	-	-	-	-	-
11.	Protein and Amino acids	+	+	+	+	+	+	+	+
12.	Anthocyanin and Betacyanin	-	-	-	-	-	-	-	-
13.	Carbohydrates	+	+	+	+	+	+	+	+
14.	Phlobatannin	-	-	-	-	-	-	-	-
15.	Flavonoids	-	-	+	-	+	+	+	+
16.	Cardiac glycosides	-	-	-	-	-	-	+	+
17.	Phenols	-	-	-	-	+	-	+	-
18.	Tannins	-	-	+	-	-	-	+	-
19.	Phytosterols	-	-	-	-	-	-	-	-
20.	Polyphenols	-	-	-	-	-	-	-	-
21.	Fixed oils and fats	-	-	-	-	-	-	+	+
22.	Fatty acids	-	-	-	-	-	-	+	+

(F) indicates Fresh; (D) indicates Dried; (+) indicates presence; (-) indicates absence

Generally, 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. The secondary phytoconstituents such as terpenoids have significant biological properties such as antimalarial, anti-ulcer, hepaticidal, antimicrobial, anti-carcinogenic and diuretic activities. The cardiac glycosides act as a cardioprotective agent. Saponins possess antimicrobial and antimalarial properties. Alkaloids which are also secondary phytoconstituents have anti-inflammatory, antioxidant, antibacterial, antifungal properties which is beneficial to human health[22]. The various other phytochemicals such as glycosides, steroids resin, quinone, flavonoids, phenols, tannins, fixed oils and fats also makes the horse gram sprouts a healthy edible product.

Quantification of the phytoconstituents

Among the fresh and dried horse gram sprouts analysed for phytoconstituents in four solvents, methanol and aqueous extracts showed prominent results both in fresh and dry samples. Hence further work was carried out only in methanol and aqueous extracts.

The total soluble sugars were quantified in the fresh and dried horse gram sprouts where the standard solution of concentration (10-100 ppm) confirmed to Beer's Law at 510nm with a regression co-efficient (R^2) = 0.9918. The plot has a slope (m) = 0.0078 and intercept = 0.0577. The equation of standard curve is y = 0.0078x + 0.0577. The results revealed that methanol and aqueous extract of fresh horse gram sprouts had 0.58 ± 0.2mg/g and 0.56 ± 0.1mg/g of glucose and the dried horse gram sprouts had 0.55 ± 0.1mg/g and 0.53 ± 0.2mg/g of glucose.

Protein content in fresh and dried horse gram sprouts samples were estimated using BSA as standard. The standard solution of concentration (100-1000 ppm) confirmed to Beer's Law at 510nm with a regression co-efficient (R^2) = 0.9901. The plot has a slope (m) = 0.0008 and intercept = 0.0614. The equation of standard curve is y = 0.0008x + 0.0614. The results indicated that methanol and aqueous extract of fresh horse gram sprouts had 37 ± 0.9mg/ml and 36 ± 1.1mg/ml of protein and the dried horse gram sprouts had 33 ± 0.7mg/ml and 32 ± 0.9mg/ml of protein.

Flavonoids estimation were carried out in fresh and dried horse gram sprouts. The quercetin solution of concentration (100-1000 ppm) confirmed to Beer's Law at 510nm with a regression co-efficient (R^2) = 0.9933. The plot has a slope (m) = 0.0008 and intercept = 0.0495. The equation of standard curve is y = 0.0008x + 0.0495. The results revealed that methanol and aqueous extract of fresh horse gram sprouts had 0.26 ± 0.11mg QE/g and 0.23 ± 0.1mg QE/g and the dried horse gram sprouts had 0.21 ± 0.1mg QE/g and 0.20 ± 0.007 mg QE/g.

Quantification of terpenoids revealed that methanol and aqueous extract of fresh horse gram sprouts had 88 ± 1.4mg/g and 86 ± 1.2mg/g and the dried horse gram sprouts had 85 ± 1.2mg/g and 81 ± 1.4mg/g of terpenoids.

Phytic acid content in the fresh and dry sprouts were estimated which showed that methanol and aqueous extract of fresh horse gram sprouts had 0.15 ± 0.005mg/g and 0.16 ± 0.007mg/g ofphytic acid and the dried horse gram sprouts had 0.17 ± 0.005mg/g and 0.18 ± 0.004mg/ of phytic acid. The phytic acid content in the fresh and dried horse gram sprouts was found to be less when compared to the control seeds used for the samples where fresh horse gram sprouts seeds had 1.4± 0.2mg/g and dried horse gram sprouts seeds had 1.5 ± 0.4mg/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 phyticacid. Chemically the carbohydrates are polyhydroxylated aldehydes or ketones and their derivatives. Carbohydrates play a major role in promoting health fitness, form a major part of food and help a great deal in building body strength, by generating energy. They are one among the three prominent macronutrients that serve as excellent energy providers, the other two being fats and proteins. Carbohydrates aid in regulating blood glucose and also take part in breaking down of fatty acids, thus preventing ketosis[23].Proteins are primary constituents made up of amino acids. Proteins are of great importance as these are highly nutritious.

More than 4000 flavonoids have been described so far within the parts of plants normally consumed by humans and approximately 650 flavones and 1030 flavanols are known. Flavonoids have broad biological and pharmacological activities. These include antimicrobial, cytotoxicity, anti-inflammatory, anti-tumour and

antioxidant properties. The terpenoids are a class of natural products which have been derived from five-carbon isoprene units. Most of the terpenoids have multi cyclic structures that differ from one another by their functional groups and basic carbon skeletons. These types of natural lipids can be found in every class of living things, and therefore considered as the largest group of natural products[24].

Legumes contain several anti-nutritional factors and one such is 'phytic acid' chemically known as 'myoinositolhexakis-dihydrogenphosphate'. It is a major storage form of organic phosphorous in cereals, legumes, oilseeds and nuts. In humans, the reduction in the digestibility of proteins, lipids and starch occurs due to the chelating property of several cations. The enzyme 'phytase' known as 'myoinositol-hexaphosphatephosphohydrolase, an acid phosphatase, have the potential to hydrolyze phytic acid to a series of lower phosphate esters of myoinositol and phosphate. The phytase enzyme contribute to food industry by decreasing the levels of phytic acid in the food and making the food safer for consuming. The phytase production is more common during sprouting which decreases the levels of phytic acid. The daily intake of phytate can be as high as 4500 mg. In average, the daily intake of phytic acid was reported to be 2000–2600 mg for vegetarian diets as well as diets of inhabitants of rural areas of developing countries and 150–1400 mg for mixed diets[25]. The results were promising with the decreased levels of phytic acid content when compared to the seeds.

Characterization by Fourier Transform Infrared Spectrophotometer (FT-IR) analysis

FT-IR 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 (Figure 3 and 4).

FT-IR 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 (Figure 5 and 6).

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 gramsprouts methanol extract had terpenoids with C-H stretch at 2930 cm^{-1} and in aqueous extract at 2928.07 cm^{-1}. The presence of different functional groups may be attributed to the existence of variety of potential phytopharmaceuticals.

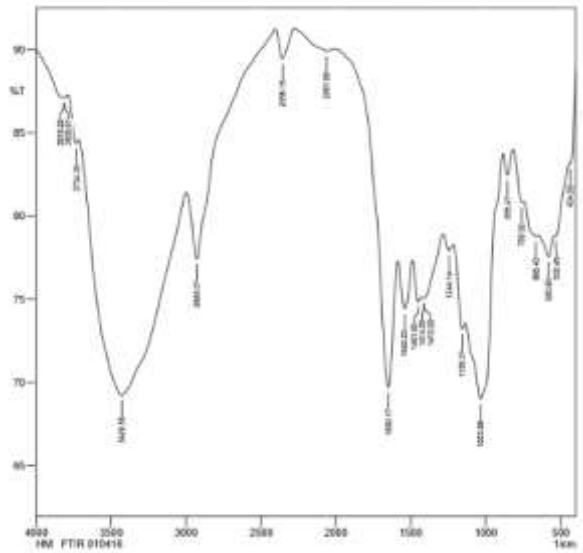

Figure 3: FT-IR spectrum of methanol extract of fresh horse gram sprouts (HM)

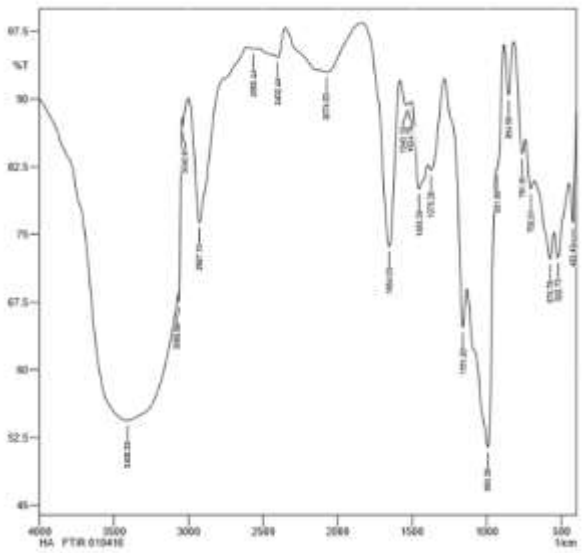

Figure 4: FT-IR spectrum of aqueous extract of fresh horse gram sprouts (HA)

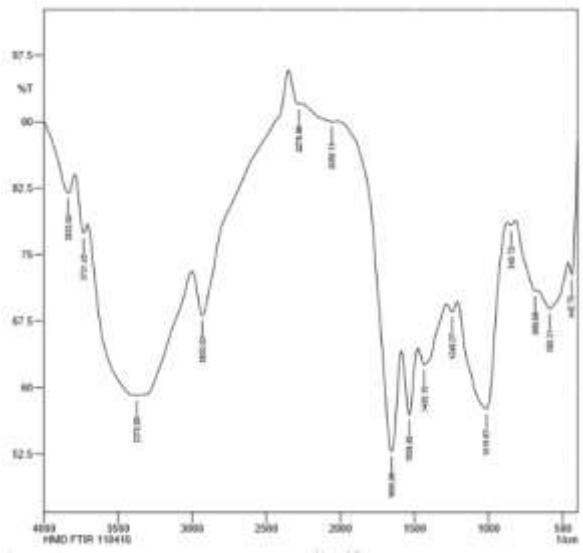

Figure 5: FT-IR spectrum of methanol extract of dried horse gram sprouts (HMD)

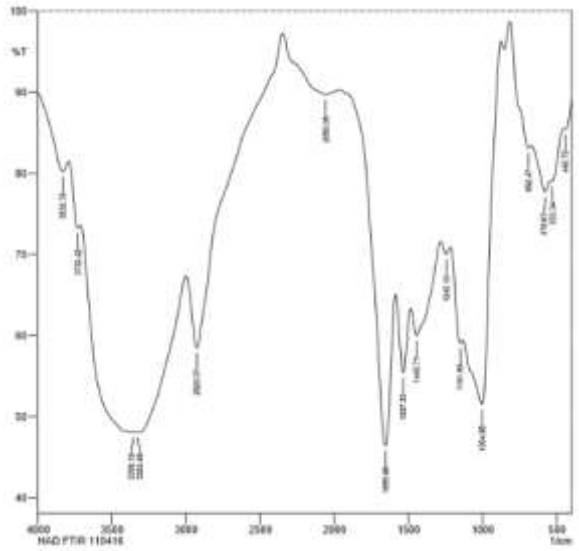

Figure 6: FT-IR spectrum of aqueous extract of dried horse gram sprouts (HAD)

Antibacterial assay

Methanol extract of fresh horse gram sprouts (HM) at 100µg showed maximum zone of inhibition of (30 ± 0.4mm) against *Shigella flexneri* and minimum zone of inhibition (5 ± 0.2mm) against *Escherichia coli*(Figure 7 and 8). Aqueous extract of fresh horse gram sprouts at 100µg (HA) showed maximum zone of inhibition (29 ± 0.7mm) against *Shigella flexneri* and minimum zone of inhibition (4 ± 0.5mm) against *Escherichia coli*(Figure 9 and 10). The minimum inhibition zone of inhibition observed against *E.coli*, reveals that the intake of horse gram sprouts will not bring down the natural microbial flora of the intestine when consumed fresh. The two extracts of dried horse gram sprouts were also tested against the human pathogens and only minimum zone of inhibition was observed whereas fresh sprouts showed maximum antibacterial activity. The presence of potent phytoconstituents like terpenoids and flavonoids might be responsible for the prominent antibacterial activity of the sprouts.

Figure 7: Maximum zone of inhibition of methanol extract of fresh horse gram sprouts (HM) against *Shigella flexneri*

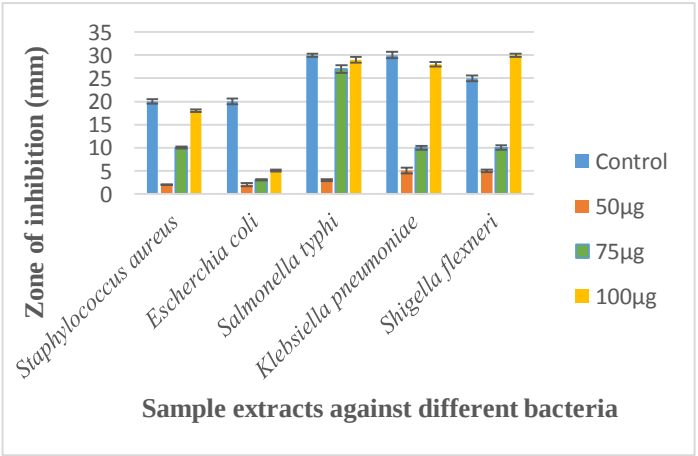

Figure 8: Antibacterial activity of methanol extract of fresh horse gram sprouts (HM) of different concentrations against different food borne pathogenic bacteria

Figure 9: Maximum zone of inhibition of aqueous extract of fresh horse gram sprouts (HA) against *Shigella flexneri*

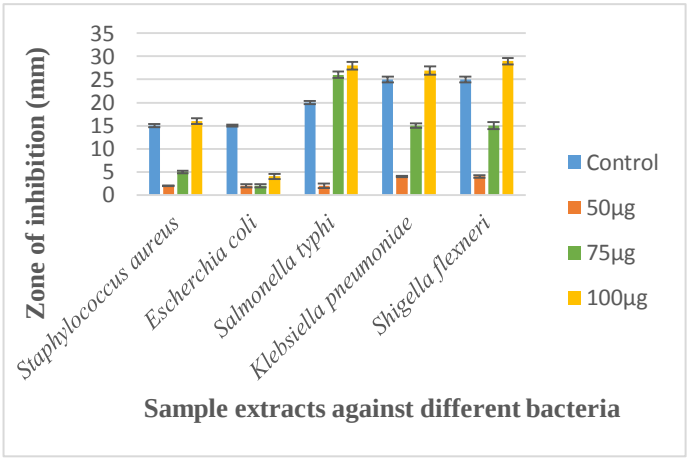

Figure 10: Antibacterial activity of aqueous extract of fresh horse gram sprouts (HA) of different concentrations against different food borne pathogenic bacteria

Food borne disorders has a major economic impact on society which causedisorders of the digestive tract. Similar studies carried out in green gram sprouts revealed maximum zone of inhibition against *Salmonella typhi*, *Klebsiellap neumoniae* and *Proteus vulgaris*[26]. Flavonoids are hydroxylated polyphenolic compounds which has a significant biological property to form complexes with soluble extracellular proteins and bacterial cell walls. Terpenoids inspite of its aromatic qualities has a strong binding affinity towards the cell wall of bacteria[27]. The results clearly showed that the sprouts can act as a potent antibacterial agent against several food borne pathogenic bacteria. The antibacterial activity of phytoconstituents present in the horse gram sprouts especially carbohydrates, proteins, terpenoids, flavonoids are mainly responsible for promoting the zone of inhibition against the human food borne pathogens. Thus consuming these sprouts as food will be highly significant in resistance towards any foodborne diseases.

In-vitro Anti-inflammatory assay

The anti-inflammatory assay was carried out at different concentrations (100, 200, 300, 400, 500µg) of methanol and aqueous extracts of fresh and dried horse gram sprouts. Results revealed inhibition of thermally induced protein (albumin) denaturation in dose dependant manner. The anti-inflammatory potential of different samples are determined based on their IC_{50} value. The IC_{50} value is the measure of the extract concentration that is required for 50% inhibition. Lesser IC_{50} value denotes the higher anti-inflammatory potential of the horse gram sprouts. The methanol extract (HM) and aqueous extract (HA) of fresh horse gram sprouts showed percent maximum inhibition of 83 ± 1.1 and 80 ± 1.2 respectively at 500µg concentration with IC_{50} value of 225.3µg/ml and 247.8µg/ml (Figure 11). The methanol extract(HMD) and aqueous extract (HAD) of dried horse gram sprouts showed percent maximum inhibition of 79 ± 1.4 and 78 ± 1.6 respectively at 500µg concentration with IC_{50} value of 250.3µg/ml and 269.7µg/ml (Figure 12).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.

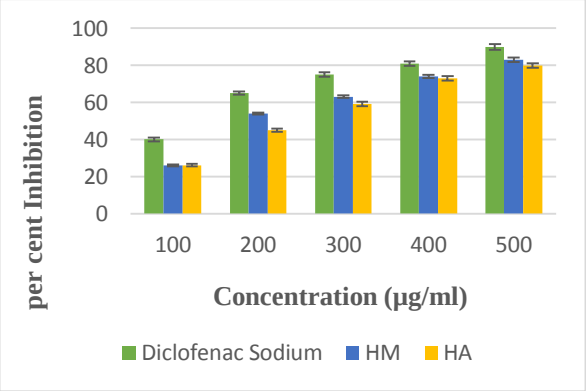

Figure 11: Anti-inflammatory activity of methanol and aqueous extract (HM and HA) of fresh horse gram sprouts

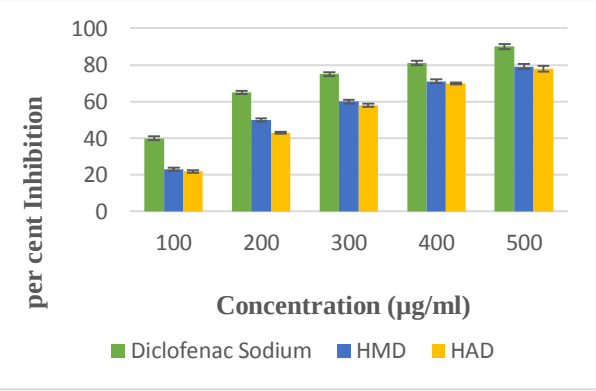

Figure 12: Anti-inflammatory activity of methanol and aqueous extract (HMD and HAD) of dried horse gram sprouts

Several harmful pathogens, irritants, stimuli, swelling and pain are some of the important causes of inflammation of vascular tissue. The prolonged inflammation leads to various diseases such as rheumatoid arthritis, hay fever, atherosclerosis. The main cause of inflammation is due to protein denaturation[28]. Agents that can prevent denaturation of proteins would be significant for development of anti-inflammatory drugs. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, organic solvent and concentrated inorganic salt or heat. Biological proteins lose their function when they are denatured. In the present study, the fresh horse gram sprouts methanol extract showed maximum anti-inflammatory activity than the dried sprouts. The results revealed that the fresh sprouts have higher anti-inflammatory potential and also have shown inhibition in a dose dependant manner indicating the inhibition of protein denaturation. The results obtained are the clear evidence for consumption of the horse gram 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* antioxidant assays**

Antioxidant assays were carried out at different concentrations (100, 200, 300, 400, 500µg) of methanol and aqueous extracts of fresh and dried horse gram sprouts. The results revealed inhibition in a dose dependant manner. The antioxidant potential were determined based on their IC_{50} value. The IC_{50} value is the measure of the extract concentration that is required for 50% inhibition. Lesser IC_{50} value denotes the higher antioxidant potential of the sprouts.

The hydrogen peroxide scavenging activity of methanol extract(HM) and aqueous extract (HA) of fresh horse gram sprouts showed percent maximum inhibition of 84 ± 1.3 and 79 ± 1.4 respectively at 500µg concentration with IC_{50} value of 229.4µg/ml and 251.1µg/ml (Figure 13) whereas the methanol extract (HMD) and aqueous extract (HAD) of dried horse gram sprouts showed percent maximum inhibition of 82 ± 1.2 and 77 ± 1.3 respectively at 500µg concentration with IC_{50} value of 243.8µg/ml and 265.9µg/ml (Figure 14).The hydrogen peroxide scavenging activity of standard ascorbic acid showed maximum percent inhibition of 90 ± 1.5% at 500µg concentration with IC_{50} value of 149.1µg/ml.

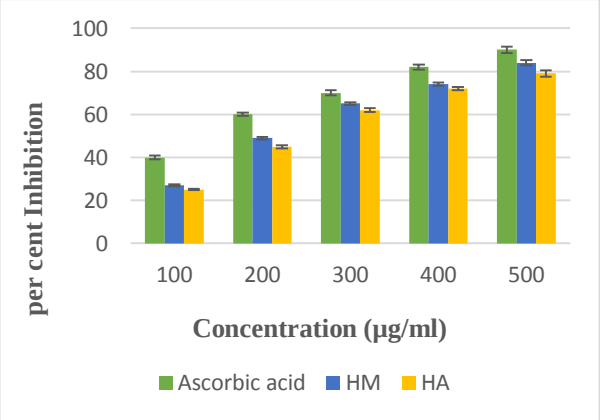

Figure 13: Hydrogen peroxide scavenging activity of methanol and aqueous extract (HM and HA) of fresh horse gram sprouts

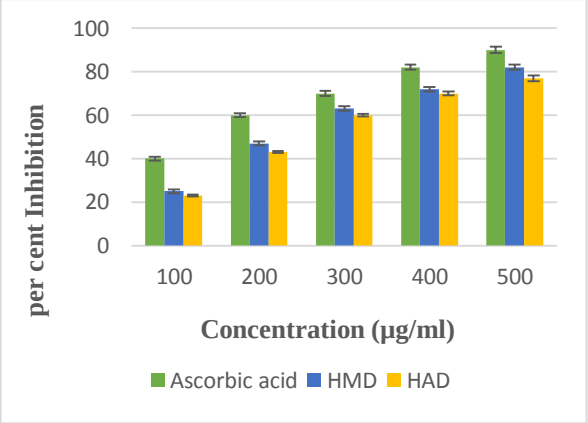

Figure 14: Hydrogen peroxide scavenging activity of methanol and aqueous extract (HMD and HAD) of dried horse gram sprouts

The reducing power activity of methanol extract (HM) and aqueous extract (HA) of fresh horse gram sprouts showed IC_{50}value of 226.3µg/ml and 265.4µg/ml (Figure 15) whereas the methanol extract (HMD) and aqueous extract (HAD) of dried horse gram sprouts showed IC_{50} value of 275.9µg/ml and 290.2µg/ml (Figure 16). The reducing power activity of standard ascorbic acid showed IC_{50}value of 149.1µg/ml.

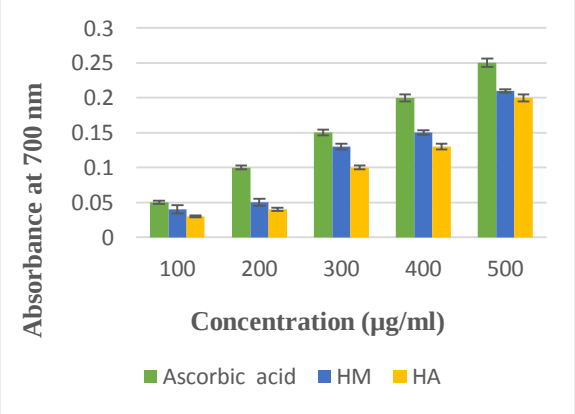

Figure 15: Reducing power activity of methanol and aqueous extract (HM and HA) of fresh horse gram sprouts

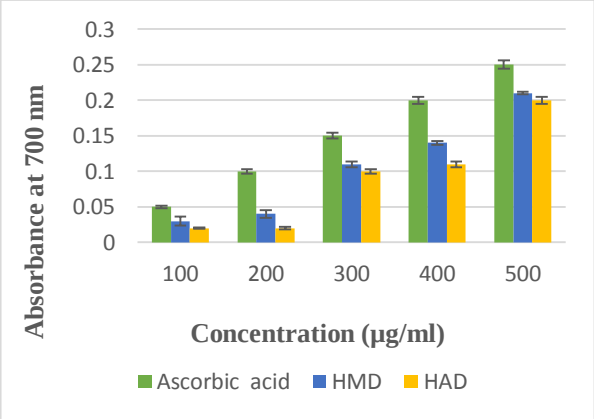

Figure 16: Reducing power activity of methanol and aqueous extract (HMD and HAD) of dried horse gram sprouts

The DPPH assay of methanol extract (HM) and aqueous extract (HA) of fresh horse gram sprouts showed percent maximum inhibition of 86 ± 1.3 and 84 ± 1.4respectively at 500µg concentration with IC_{50}value of 180.9µg/ml and 193.3µg/ml (Figure 17) whereas the methanol extract (HMD) and aqueous extract (HAD) of dried horse gram sprouts showed percent maximum inhibition of 84 ± 1.3 and 82 ± 1.4 respectively at 500µg concentration with IC_{50} value of 206.3µg/ml and 219.3µg/ml (Figure 18). The DPPH scavenging activity of standard ascorbic acid showed percent maximum inhibition of 90 ± 1.5 at 500µg concentration with IC_{50}value of 149.1µg/ml.

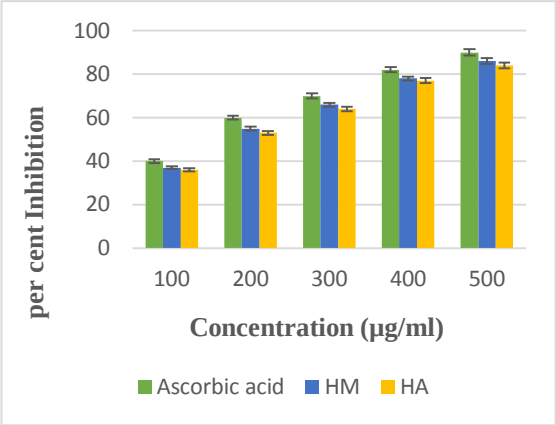

Figure 17: DPPH free radical scavenging activity of methanol and aqueous extract (HM and HA) of fresh horse gram sprouts

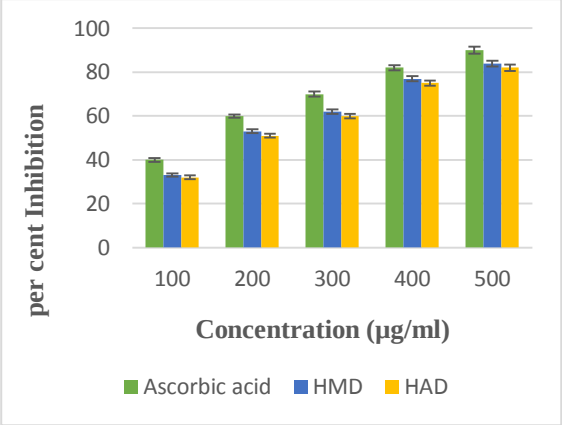

Figure 18: DPPH free radical scavenging activity of methanol and aqueous extract (HMD and HAD) of dried horse gram sprouts

Antioxidants are molecules capable of suppressing the oxidation of other molecules. Oxidation is a chemical reaction that transfers the electrons from a substance to a particular oxidizing agent. Oxidation reaction produce free radicals which in turn produce chain reactions that damage the cells. Antioxidants terminate these chain reactions by removing the free radical intermediates thereby inhibiting other oxidation reactions. Oxidative damage have a significant role in several human diseases like cancer, atherosclerosis and arthritis[29]. Plant antioxidants are composed of different substances like ascorbic acid, tocopherols, terpenoids. Among the three antioxidant assays carried out in fresh and dried horse gram sprouts, DPPH scavenging assay indicated a prominent antioxidant activity when compared to hydrogen peroxide scavenging assay and reducing power assay. Thus it clearly shows that the horse gram sprouts can be a potent natural antioxidant agents due to the presence of rich phytoconstituents specifically terpenoids and flavonoids.

Terpenoids provides a measure of protection against several diseases, especially those related to chronic damage and growth dysregulation. These have a unique antioxidant activity in their interaction with free radicals. These react with free radicals by partitioning themselves into fatty membranes by virtue of their long carbon side chain. Terpenoids are unsaturated compounds having one or more double bonds which undergo addition reactions with hydrogen, halogens, halogenic acids which results in formation of hydrates. They also form characteristic addition products with NO_2, NOCl and NOBr. These addition products are mainly responsible for the potentbiological properties of the terpenoids[30]. Flavonoids are large class of benzo-pyrone deriatives, the antiradical activity of flavonoids is directed mostly towards hydroxyl, superoxide as well as peroxyl and alkoxyl radicals which possess multiple activities like antibacterial, anti-inflammatory, immune-stimulating, anti-allergic, vasodilatory and estrogenic effects. These biological properties are said to be related to their antioxidative properties.

Gas Chromatography Mass Spectrometry (GC-MS) analysis

GC-MS is one of the technique 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, geranylgeraniol, gamma-sitosterol, fumaric acid and gamma tocopherol(Figure 19 and Table 3).Most of the compounds were grouped under terpenoids, fatty acids, carbohydrates and amino acids. 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 phytopharmaceuticals reported.

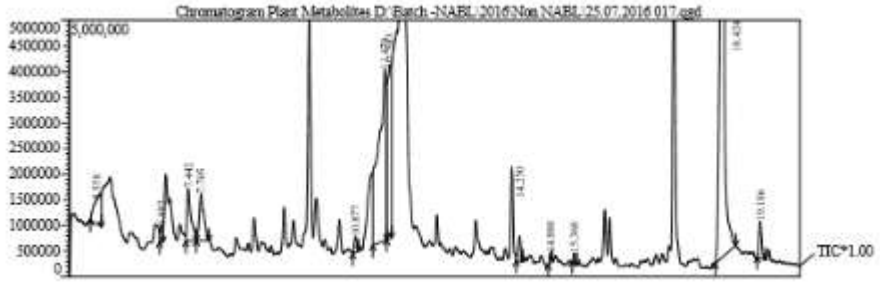

Figure 19: GC-MS spectrum of methanol extract of fresh horse gram sprouts (HM)

Table 3: GC-MS analysis of methanol extract of fresh horse gram sprouts (HM)

Peak	R. Time	Name	Area	Area %	Height	Height %
1	5.558	Diglycerol	5553189	1.72	466880	1.05
2	6.892	DL-Proline	1198693	0.37	311047	0.70
3	7.442	DL-Phenylalanine	5850439	1.82	1023951	2.30
4	7.705	1,3-Propanediol	6079655	1.89	908074	2.04
5	10.877	Quinoline	1228548	0.38	317104	0.71
6	11.479	Vinyl caprylate	37625325	11.69	3333966	7.48
7	11.583	Beta-D-Mannofuranoside	19479357	6.05	3375609	7.57
8	14.250	Myo-Inositol	1727775	0.54	485447	1.09
9	14.880	Isopropyl Myristate	585072	0.18	203905	0.46
10	15.368	Oxirane	586868	0.18	189205	0.42
11	18.424	Ascorbic acid	69592194	21.62	12287368	27.56
12	19.186	n-Nonadecanol-1	2585400	0.80	723170	1.62
13	23.172	Cis-Vaccenic acid	42685717	13.26	5768998	12.94
14	24.600	1-Heptacosanol	573755	0.18	180289	0.40
15	26.830	Gamma-Linolenic acid, methyl ester	1198640	0.37	299235	0.67
16	42.775	Glycerol tricaprylate	843306	0.26	218510	0.49
17	43.183	Stigmasterol	6660263	2.07	966317	2.17
18	43.656	Geranylgeraniol	2927669	0.91	1172076	2.63
19	44.666	Gamma-Sitosterol	33753319	10.48	5060335	11.35
20	45.225	Fumaric acid	22995702	7.14	1965061	4.41
21	46.046	Gamma-Tocopherol	58204099	18.08	5322475	11.94
			321934985	100.00	44579022	100.00

***Insilico* docking studies**

GC-MS studies confirmed the presence of terpenoids, fatty acids, carbohydrates, amino acids and other small functional groups. Thus from the GC-MS analysis, screening was carried out for the therapeutic compounds related to anti-ulcer property. DL-Proline, a free amino acid produced from methanol extract of fresh horse gram sproutstend to have anti-ulcer property. Diclofenac sodium was used as standard. Diclofenac is a non-steroidal anti-inflammatory agent which is primarily available as the sodium salt (Pubchem database).

Peptic ulcer is caused by *Helicobacter pylori*, a gram-negative bacillus or some allergic reactions to medicines like non-steroidal anti-inflammatory drugs found in the digestive tract in the stomach or the duodenum. The pathogenic activity is found in the Type-I strains of the bacteria which encodes the effector protein cytotoxin-associated gene (cagA)[31]. The bacteria entering the host cell, cagA affects the shape of the cell, increases the cell motility, intrupt the cell junctional activity which results in gastric carcinomas and ulcers.

The target protein of *Helicobacter pylori*, was obtained from Protein Data Bank (http://www.rcsb.org/pdb/)- PDB ID: 1G60. The bioactive compound docked against the target protein in mcule database showed the anti-ulcer property of the compound through docking scores. More negative values

are indication of higher binding affinity which clearly indicates the strong anti-ulcer property. Docking analysis of DL-Proline from fresh horse gram sprouts methanol extract (HM) showed docking scores of -4.7, -4.5, -4.2 and -4.1 (Figure 20). Docking analysis of standard diclofenac sodium showed docking scores of -7.5, -7.3, -7.1 and -6.8 (Figure 21). Horse gram sprouts showedprominent binding affinity against *Helicobacter pylori* when compared with the standard drug compound diclofenac sodium. The docking results are a clear evidence for the sprouts having potent anti-ulcer property. Thus the horse gram sprouts with enriched phytopharmaceuticals can be recommended as a natural edible product for ulcers.

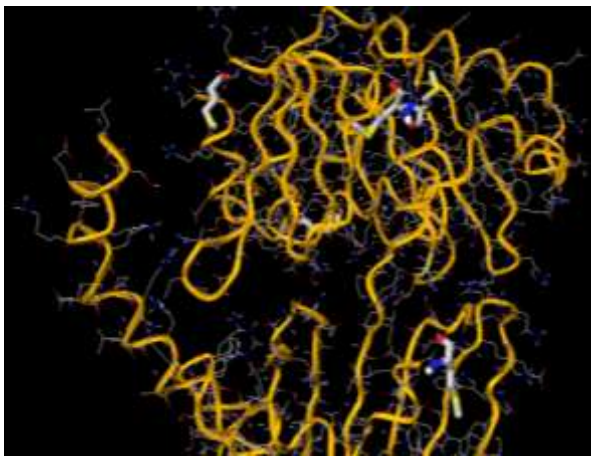

Figure 20.Illustration of DL-Prolinefrom methanol extract of fresh horse gram sprouts(HM) docked with target protein 1G60- *Helicobacter pylori*

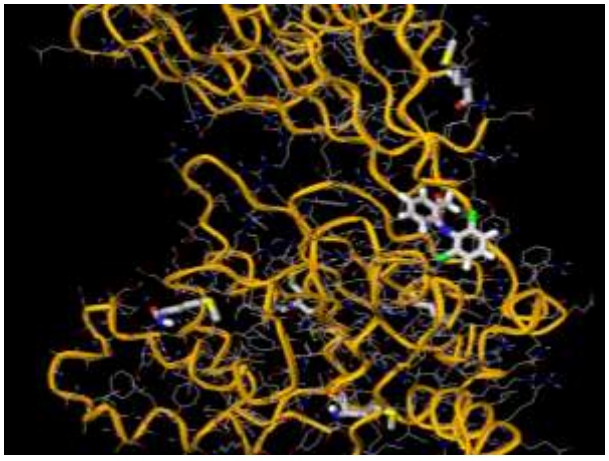

Figure 21. Illustration of standard Diclofenac sodium docked with target protein 1G60- *Helicobacter pylori*

Conclusion

Fresh and dried sprouts of *Macrotyloma uniflorum* revealed a wide spectrum of potential phytopharmaceuticals possessing antibacterial, anti-inflammatory an antioxidant activities. GC- MS studies indicated the specific phytoconstituents. Further, the *in-silico* analysis confirmed thepresence of DL-Proline, a strong amino acid having anti-ulcer property against the bacterium,*Helicobacter pylori*. Thus, thestudy confirms the presence of highly rich total soluble sugars, proteins, flavonoids and terpenoids in the sprout extracts. The present workemphasis the potent secondary phytoconstituents of the horse gram sprouts, which could be recommended for human consumption. The dried form can also be used as nutraceuticals with a quality check which could be further involved in the isolation, purification and characterization of the effective phytopharmaceuticals for the development of novel drugs.

Conflicts of interests

The authors declared that they had no conflicts of interests.

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