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Formulation and phytochemical evaluation of nutritional product containing Job's tears (*Coix lachryma-Jobi* L.)

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Abstract
Coix lachryma-Jobi L., commonly named Job's tears seeds cultivated in the north-eastern parts of India, China, Philippines, Burma, Sri Lanka and some other Asian countries are taken into consideration for this study. The seed flour was extracted with ethanol whereby the extract was subjected to different phytochemical tests. The phytochemical tests included Phenols estimation, Flavanol content and antioxidant activity and tannin content of the seeds and gave good results. Job's tears was found to be a very rich source of flavanoids. The antibacterial activity of the extract was also tested which showed no considerable results. The seeds were further used to formulate a daily nutritional product by adding additional materials such as- Ragi flour (*Eleusine coracana*), Giloyi stem powder (*Tinospora Cordifolia*), sugar, coconut powder and whey protein. Malting of the seeds was also done, but not more than 50% seeds showed germination. Another product was formulated using the malted seeds. The products were subjected to nutritional analysis, giving high amount of proteins in the malted product, while the former can be considered as a good source of dietary fibers and fat. Further the carbohydrate content and energy value were also calculated. As a result, for malting there was a significant increase in the protein quantity and the taste was also better. The GC-MS screening showed the presence of several useful compounds.

Keywords: Antioxidant, Phytochemical, Phenol, Flavanol, Antibacterial, Malting, GC-MS.

1. Introduction
Is it just about "survival" or "healthy survival" would be more appropriate? Looking at our eating habits and the increasing number of diseases native to the human body the question makes sense. Developing countries are facing the problem of under- nutrition while the developed one's facing a deficiency of micro- nutrients. Healthy food alternatives included in the daily diet is the need of the hour whereby starting with cereals. Cereals cover the largest part of our diet. Kernels and endosperm of cereal grains are a rich source of food carbohydrates. They are rich in vitamins, minerals and essential amino acids. The non starch polysaccharides present in the cereals help in reducing blood cholesterol levels and reduce the risk of cancer [1].

One of such cereal grains is *Coix lachryma-Jobi* L., commonly named Job's tears. It is native to China, but also cultivated widely in many other Asian countries such as Philippine, Burma, Sri Lanka and Thailand [2]. The grain is having a hard dark brown to gray colored shell/ hull and is pear shaped [3].

Job's tears has been widely studied and reported to contain thiamine, riboflavin, niacin and ascorbic acid [2], comprises of 10.83% moisture, 13.05 g protein (protein content approximately double to that of rice), 5.45 g fat, 68.60 g carbohydrate, 0.36 g fiber and 1.3 g ash and Job's tears flour contained 2.25% amylase [4, 5]. The major protein is coixin whih is rich in proline and leucine but poor in lysine [6]. The grain is used in Chinese herbal medicines for long in treatment of diseases related to spleen and kidney [7], in treatments for a syndrome of "thoracic paralysis [8], utilized in a traditional formula for treating fluid problems [9], in treating damp heat [10]. In the book "Chinese Medicinal Teas" a recipe for the wind-damp syndrome with slight heat and swelling is given [11]. It has been used by the women in South-East Asia and Japan for beautifying skin. Coix has been widely used in the post cancer treatments and as a preventive measure of the disease because of the two components isolated namely: coixol and coixenolide [12]. As indicated in the book Anticancer Medicinal Herbs a daily dose of jobs tears along with glutinous rice is recommended in the stomach and larynx cancer [13]. Studies on the Anticancer properties of the seed include- Neutral lipids isolated from the endosperm may be prevented against pancreatic cancer [14]; The methanolic extract of adlay seeds reduced the PGE (2) level in serum and inhibited COX-2 expression of tumor tissues in nude mice [15]; the components of adlay seed exert an anticancer effect in vitro and in vivo

and may be useful for the prevention of lung tumorigenesis [16]; Five active compounds that inhibit cancer cells were isolated from adlay bran [17]; Traditional Chinese Medicine-based cancer treatment affects cellular pathways of recognized importance in neoplasia [18]. Administration of adlay may be considered for therapies targeting obesity [19, 20]. Further biomedical bnefits of this grain include antiproliferative and chemopreventive effects on lung cancer, antitumor, anti-allergic, inducing embryotoxicity and fibrinolytic activities [21-25]. Recent trials on humans and animals have shown it to be active in improving the lipid metabolism [26, 27]. May it be due to the water soluble polysaccharides and oil present [28]. Extraction of FOL (fructo oligosaccharides) makes it possible to be used as a prebiotic. Fermentation of Job's tears with *Lactobacillus* in soy milk has been reported to increase the immunological effects in wistar rats [29].

The flour is high in fat, which is a limitation to its uses. However, some alternative is needed to de-fat the flour to be used further. Though potential food applications include-adition in soups, popular dessert made by adding jobs tears mixed with sugar and coconut milk in Thailand, fermented grains are used in making beer and wine [30], feeding poultry [31], We have used this flour with some other ingredients such as-giloyi, whey protein, roasted ragi flour, sugar and coconut to make a daily formulation that may help reduce heart diseases, risk of cancer, beneficial for glowing skin, strengthens liver, improves digestion and providing overall health benefits. The formulation is appropriate for adult human, but not fit for pregnant women as Job's tears is considered toxic for the growing embryo. The phytochemical investigation in addition to the malting of jobs tears (adlay) grains was also undertaken during the study.

(A). Job's tears seeds with seed coat

(B). Job's tears seeds without seed coat

2. Materials and Methods

2.1 Preparation of grains

The Jobs tears grains were brought from NBPGR, Umiam, Meghalaya. The grains appeared to be variation in color from dark gray to light and white. The shell was hard so was grinded in grinder first and then subjected to manual handling to remove hard partitions and obtain the seeds. Some seeds were kept aside for malting. Remaining seeds were then subjected to milling for the removal of the bran and to obtain flour.

2.2 Preparation of extracts

The flour (50 gm) was soaked into 200ml ethanol for 2 days in an incubator shaker (at 50 °C) to obtain the ethanol extract. The solution was filtered and the filtrate was subjected to drying in oven at 70 °C so as to remove all the extraction solvent. The extract obtained was stored in refrigerator at 4 °C until further use.

2.3 Malting

The grains were steeped in water for 72 hrs with replacing water after an interval of 16 hrs. Germination was seen and germinated grains were subjected to rest in a wet muslin cloth for another 8 hrs. After this the grains were sun dried for 10 hrs followed by oven drying at 40 °C for 1 day. The germinated pods were removed manually as they are toxic. Malting enhances the level of certain phytochemicals, proteins, etc. hence increasing the overall nutritional quality of the grains.

2.4 Antioxidant activity

2.4.1 DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

The antioxidant efficacy of the extract was measured by the DPPH (*1,1-diphenyl-2-picrylhydrazyl)* radical scavenging process [32]. DPPH is a nitrogen centered, stable free radical having maximum absorption at 517 nm in alcoholic solution. 1ml of the prepared extracts was added to 1ml 0.3 mM DPPH (1.1829 mg in 10ml methanol) and 1ml methanol and a blank was prepared by just adding DDPH, distilled water and methanol 1ml each. All the solutions were kept in dark for 10 mins. OD was measured at absorbance of 517 nm. %inhibition was calculated by the following formulae:

$$\% \text{ inhibition} = [(B-A)/B] \times 100$$

Where B= Absorbance of blank solution,
A= Absorbance of sample

2.4.2 FRAP Assay

FRAP stands for ferric reducing antioxidant power [33]. FRAP comprises of 3 reagents A, B, C prepared. Reagent A comprises of 3.1 gm sodium acetate added to 16ml glacial acetic acid and volume made upto 100ml followed by freezing for 4-5 hrs. Reagent B is a TPTZ solution in HCl, 40 mM HCl was prepared followed by the addition of 0.0781 gm TPTZ in 25ml HCl. Heat the solution at 50 °C. Reagent C is light sensitive, i.e. 20 mM $FeCl_3.6H_2O$. FRAP is made by adding reagent A, B, C in the ratio 1:1:10. 200µl sample added to 1800µl FRAP incubated for 4 mins at RT and the absorbance measured at 593 nm.

2.5 Phytochemical Analysis

2.5.1 Total flavanoid content using UV-Vis calorimetric method

Total flavanoid content in the ethanol extract was determined by the UV-Visible spectrophotometry [34]. The extract was dissolved in DMSO. Out of this 250µl extract was added to 4.5ml distilled water and 0.3ml 5% $NaNO_2$ and kept for 5 mins. Added 0.3ml 10% $AlCl_3$ and incubate for 6 mins. Add 2ml 1 M NaOH to it and make up the final volume to 10ml with distilled water. Vortex all the test tubes and take OD at 510 nm. A 5-points calibration curve was made with Rutin as standard.

2.5.2 Total Phenol estimation by FC method

The method was given by Slinkard & Singleton [35]. 100µl of sample was added to 250µl FC reagent with a further addition of 1.15ml distilled water. Vortex the test tubes and added 1.5ml 20% sodium carbonate, incubation for 2 hrs add 2ml distilled water followed by measuring the OD at 765 nm. Standard curve generated using Gallic acid as standard.

2.5.3 Total Tannins estimation

Estimation of tannin content of the flower was done by the method of Van Buren (1981) [36]. To 1 gm sample 100ml water was added and allowed to shake for 1 hr in an incubator shaker.

The solution was filtered and 5ml filtrate was taken in a test tube. To this 1ml of 0.1 M $FeCl_3$ made by dissolving it in 0.1N HCl was added with further addition of 1ml 0.008 M KCN, OD was measured at 615 nm within 10 mins. A standard curve was generated using tannic acid as standard.

2.5.4 Crude alkaloid content

Alkaloids were determined by gravimetric method of Harborne (1973) [37]. 5 gm sample was added to 10% ammonium hydroxide stirred and allowed to stand for 4hrs and filtered. The filtrate was evaporated to $1/4^{th}$ of the original volume on a hot plate. To this conc. NH_4OH was added drop wise in order to precipitate the alkaloids. The precipitates were filtered using a pre weighed filter paper and washed with 10% ammonium hydroxide solution. The precipitates were dried with the filter paper in an oven for 30 mins at 60 °C and reweighed. The amount of alkaloids present was calculated in % alkaloid in the sample by the formulae:

$$\% \text{ Alkaloid} = \frac{W_2 - W_1}{W} \times 100$$

Where:
W_1- weight of the tissue paper
W_2- weight of the tissue with alkaloids
W- Weight of sample

2.6 Antibacterial activity

In order to determine antibacterial activity of ethanolic extract, Agar well diffusion assay was performed. Antibacterial activity was tested against *Bacillus subtilis* (gram +ve), *Staphylococcus epidermidis* (gram +ve) and *Escherichia coli* (gram -ve), *Salmonella enterica* (gram -ve) and *Bacillus cereus* (gram +ve). The test bacteria were grown in sterile Nutrient broth tubes respectively. The broth cultures of bacteria were then aseptically transferred to the agar plates by pour plate method. Wells of 6 mm diameter were created in the inoculated plates using sterile cork borer. Different concentrations of the extracts were prepared by dissolving in DMSO (100 mg and 50 mg in 1ml DMSO) and were filled in labelled wells. The plates were incubated at 37 °C for 24 hours and the zone of inhibition was measured.

Methanol and DMSO were separately plated and used as a control for the experiment.

2.7 GC-MS Analysis (Determination of secondary metabolites)

The secondary metabolites in the sample were identified by the GC-MS method. For this 1µl of sample extract added to the respective solvent was analyzed. As the name suggests Gas Chromatography- Mass Spectrometry, so the carrier gas here is helium. An Agilent 6890 GC with 5975B mass spectrometric detector (MSD) was used in the scan mode (m/z 35-1050) for all samples. Screening of volatiles and semi volatiles were performed using the automatic RTL screener software in combination with the Agilent NIST'05 library. The detected compounds have been identified by the NIST'05 mass spectrum library.

2.8 Formulation

The formulation was done by adding healthy alternatives to Job's tears as previous studies and reports suggest that the

grain should not be consumed alone. Two formulations were made:

1. Formulation 1: 50% Job's tears flour, 20% ragi flour, 10% coconut powder, 5% whey protein 15% sugar and a small amount of giloyi stem powder (so that the product did not give bitter taste app. 0.5%).
2. Formulation 2: 20% Job's tears flour, 30% malted job's tears flour, 10% coconut powder, 5% whey protein 15% sugar and 0.5% giloyi stem powder.

2.9 Nutritional analysis of the formulations

All the nutritional analyses were done under the methods given by AOAC (Association for Applied Chemists). The total moisture and ash content by the AOAC method Ref. 942.05 respectively [38]. The total protein content using the Kjeldahl method Ref. 976.05 (AOAC, 1990) [39]. A gravimetric method was used for determination of total dietary fiber after the enzymatic digestion of starch and protein in fat and moisture free sample (AOAC, 1990) [40]. Crude fat content was determined by extracting the sample in petroleum ether followed by evaporation and weighing while the total carbohydrate content was also measured. Minerals, trace elements and heavy metals in the examined material were determined by using Optima 2100 DV ICP-OES (Perkin-Elmer, USA), after prior mineralization in an Anton Paar Multiwave microwave digester (Anton Paar Ltd., Hertford, UK) as per Ref 956.52 (AOAC, 2005). As a standard, the certified multi element standard solution ''ICP Multi-element Standard IV" (Merck, Darmstadt, Germany) was used for the instrument's response.

3. Result and Discussions

3.1 Nutritional Analysis

Nutritional profiling of both the formulation reveals that the products are a healthy source of nutrition in all respects. Moisture and dry matter (ash) content is very important to determine because they directly affect the stability and storage of food. From the results we can interpret that malting increases the protein content of food. Hence formulation 1 is a good source of energy, but formulation 2 is better health alternative due to higher protein content and less fat content. The results are given in table 1.

Table 1: Nutritional profiling of Formulations.

Nutritional attributes	Formulation 1	Formulation 2
Moisture content	9.1 %	9.0 %
Aish content	6.07 %	6.04 %
Protein content	11.75 %	13.85 %
Dietary fibre content	8.26 %	6.60 %
Fat content	9.1%	5.31 %
Carbohydrate content	56.49 g/ 100 g	58.9 g/ 100g
Energy value	319.01 Kcal	303.02 Kcal

The mineral composition of raw sample of jobs tears were analysed by ICP-OES. The results revealed that the formulation contains high amount of Calcium, magnesium and phosphorus. Calcium is very good for bones, helps in prevention of osteoporosis and fractures [41]; phosphorus also helps in healthy bone formation, helps in repairing cells and tissues, it improves digestion, keeps the hormonal balance, etc. Magnesium on the other hand enables nerves to function and helps creating energy out of food. Some recent studies prove it to be helpful in reducing the risk of heart diseases, hypertension and diabetes [42-44]. Results are given in table 2.

Table 2: Mineral content estimation of raw Job's tears flour.

Analyte	Concentration (µg/g)
As	ND
Be	ND
Ca	125.00
Cd	ND
Co	ND
Cr	1.00
Cu	2.50
Fe	46.25
Li	ND
Mg	735.00
Mn	25.00
Mo	ND
Ni	ND
P	20325.00
Pb	ND
Sb	ND
Se	ND
Sn	ND
Sr	ND
Ti	ND
Tl	ND
V	ND
Zn	ND

3.2 Phytochemical analysis

Polyphenolic substances are the major category of phytochemicals found in higher plants are the cause behind the antioxidant attributes. The uptake of these compounds is an important health-protecting agent. These bioactive compounds retards or inhibit lipid autoxidation by acting as radical scavengers and, therefore, are essential antioxidants that protect against the propagation of the oxidative chain. Grounds for their use in the prevention of degenerative diseases are coming forth. Observational studies of animal and human cell lines have shown that polyphenols can play a part in preventing cancer and cardiovascular diseases, when consumed daily in equal amounts. The important classes analyzed here were phenol, flavanol, tannins and alkaloids performing similar functions. The results are given in table 3.

Table 3: Results for phytochemical analysis

Analyte (Ethanol extract)	Content
Total Phenols	20.5 mg GAE/ gm sample
Total Flavanoids	11.5 mg RE/ gm sample
Crude Alkaloid content	5.38%
Total Tannins	17.5 mg TAE/gm sample

3.3 Antioxidant Activity

3.3.1 DPPH radical scavenging assay

DPPH is nitrogen centred; stable free radical having maximum absorption at 517 nm in alcoholic solution. It becomes a stable diamagnetic molecule on accepting an electron or hydrogen atom [45]. In the presence of an extract capable of donating a hydrogen atom, the free radical nature of DPPH is lost and the purple color changes to yellow (diphenylpicrylhydrazine). The bleaching of DPPH radical is one of the most widely used strategies to evaluate the antioxidant activity of extracts. The results are given in table 4

3.3.2 FRAP assay

The method described measures the ferric reducing antioxidant power. In acidic medium the ferric–trotripyridyltriazine (FeIII-TPTZ) complex is reduced to ferrous (FeII) form and an intense blue color with an absorption maximum at 593 nm develops. However Job's tears ethanol extract showed a very good antioxidant activity against DPPH free radical. The caliberation curve revealed highly positive linear relation between FRAP values and BHT standard. The results are given in table 4.

Table 4: Antioxidant activity of Job's tears ethanol extract.

Test	Antioxidant activity
DPPH scavenging assay	91.35% inhibition
FRAP assay	270.25 µg BE/mg sample

3.4 Secondary metabolites estimation (GC-MS)

The Job's tears extract was dissolved in ethanol and petroleum ether and both were subjected to GC-MS analysis. Where the data for A means extract dissolved in ethanol and data for B means extract dissolved in petroleum ether. Some of the important compounds detected in the ethanol fraction include – 1-Heptadecene, Tetradecanoic acid, Trichloroacetic acid, tetradecyl ester, Trichloroacetic acid, pentadecyl ester, n-Hexadecanoic acid, 9-Octadecenoic acid (Z)-, methyl ester, 9,12-Octadecadienoic acid (Z,Z)-7-Pentadecyne, 2,6,10,14,18,22 Tetracosahexaene,2,6,10,15,19,23-examethyl-, Squalene, Ergost-5-en-3-ol, (3.beta.) Campesterol, gamma.-Sitosterol, beta.-Sitosterol , Stigmasterol, 22,23-dihydro which posess antioxidant activities and health improving functions in the body.

Table 5: GC-MS analysis of Job's tears extract in Ethanol solvent

Compound Name	Cas#	RT	% Area
1-Heptadecene	006765-39-5	16.164	0.27
Tetradecanoic acid	000544-63-8	18.183	0.13
Tetradecanoic acid	000544-63-8	18.183	0.13
Trichloroacetic acid, tetradecyl ester	074339-52-9	18.429	0.10
Trichloroacetic acid, pentadecyl ester	074339-53-0	18.429	0.10
n-Hexadecanoic acid	000057-10-3	20.325	31.56
9-Octadecenoic acid (Z)-, methyl ester	000112-62-9	21.502	1.28
9,12-Octadecadienoic acid (Z,Z)- 1-Hexadecyne	000060-33-3	22.086	46.05
9,12-Octadecadienoic acid (Z,Z)	000060-33-3	23.140	0.90
E,E-10,12-Hexadecadien-1-ol acetate	1000130-87-6	23.532	2.36
9,12-Octadecadienoic acid (Z,Z)-7-Pentadecyne	000060-33-3	26.482	4.58
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl	000111-02-4	27.559	2.34
Squalene	000111-02-4	27.559	2.34
Ergost-5-en-3-ol, (3.beta.)-Campesterol	004651-51-8	33.402	1.18
gamma.-Sitosterol	000083-47-6	35.353	2.27
beta.-Sitosterol	000083-47-6	35.353	2.27
Stigmasterol, 22,23-dihydro	1000214-20-7	35.353	2.27

Table 6: GC-MS analysis of Job's tears extract in petroleum ether solvent

Compound Name	Cas#	RT	% Area
n-Hexadecanoic acid	000057-10-3	20.325	2.96
9,17-Octadecadienal, (Z)	056554-35-9	21.985	16.94
9,12-Octadecadienoic acid (Z,Z)	056554-35-9	21.985	16.94

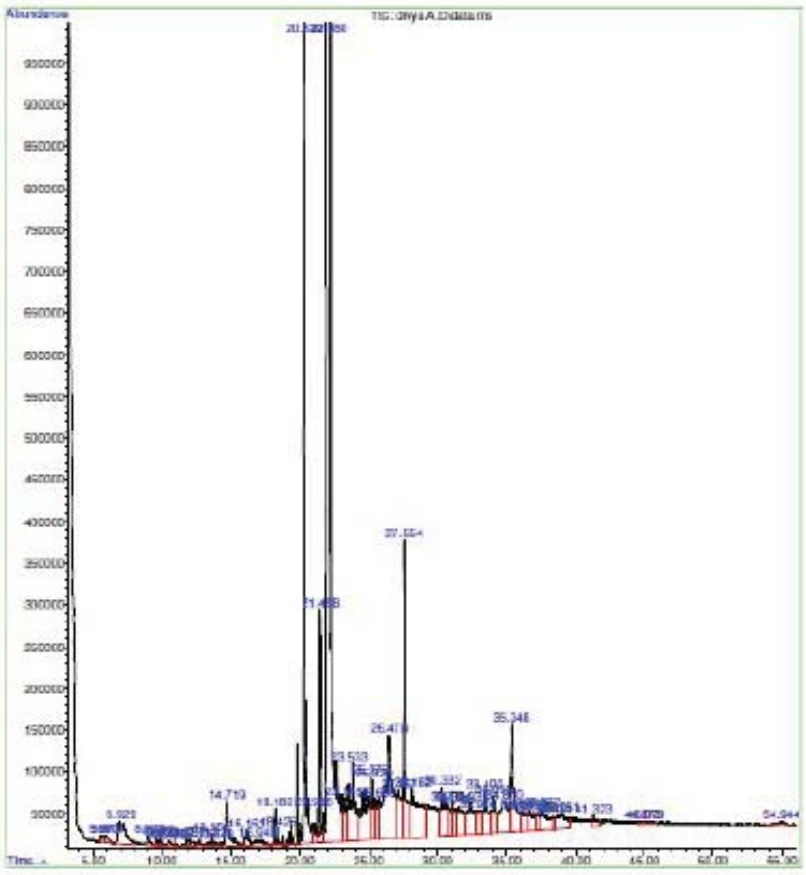

Fig 1: GC-MS chromatogram for ethanol extract of Job's tears seeds.

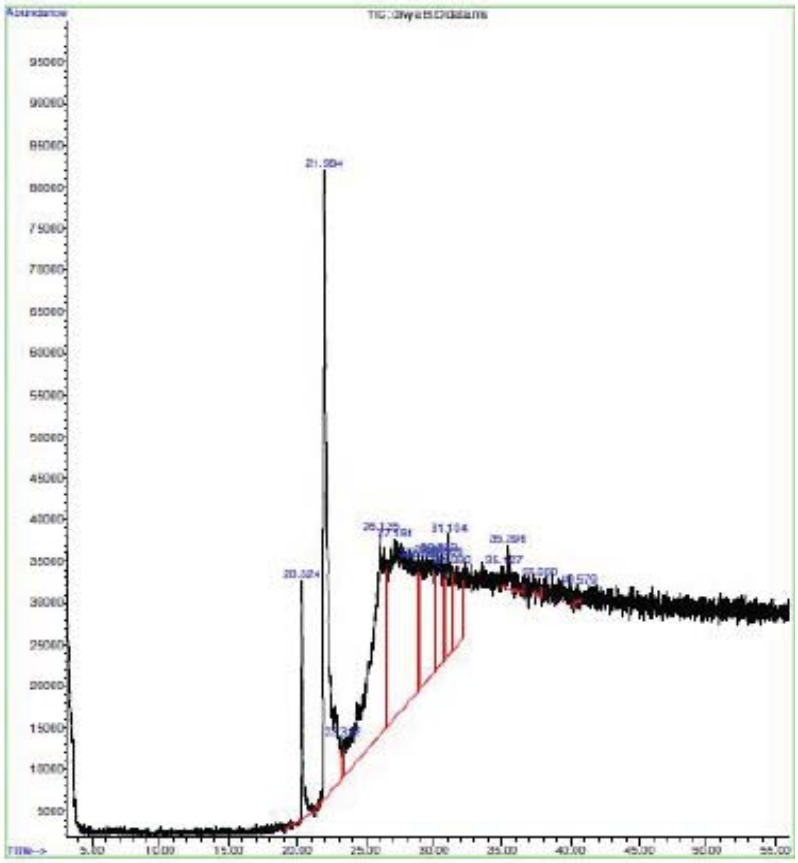

Fig 2: GC-MS chromatogram for petroleum ether extract of Job's tears.

3.5 Antibacterial activity

Antibacterial activity of the extract was tested against 3 gram +ve strains *Bacillus subtilis*, *Staphylococcus epidermidis* and *Bacillus cereus* and 2 gram –ve strains namely *Escherichia coli* and *Salmonella enterica.* The extract was dissolved in DMSO and was kept as control also. Job's tears was found to be ineffective against *Salmonella enteric* and does not posess very good antibacterial power. The table 7 shows the zone of inhibition of extract against the bacterial strains.

3.6 Sensory evaluation of the product

Sensory Evaluation is defined as "A scientific discipline used to evoke, measure, analyze, and interpret those responses to products that are perceived by the senses of sight, smell, touch, taste and hearing (Stone and Sidel 1993) [46]." The sensory quality of a food product is the single most important factor influencing its success in the marketplace. The products sensory evaluation was done by 20 different candidates and found to have a good overall acceptability. However the formulation containing the malted Job's tears tasted better, rest all the aspects were approximately same.

Table 7: Antibacterial activity of Job's tears extract

Bacterial strain	Zone of inhibition (in cm)	
	Extract conc (100 mg)	Extract conc (50 mg)
Bacillus subtilis	**1.4**	**0.8**
Staphylococcus epidermidis	**0.9**	-
Bacillus cereus	**1.0**	**0.9**
Escherichia coli	-	**1.0**
Salmonella enterica	-	-

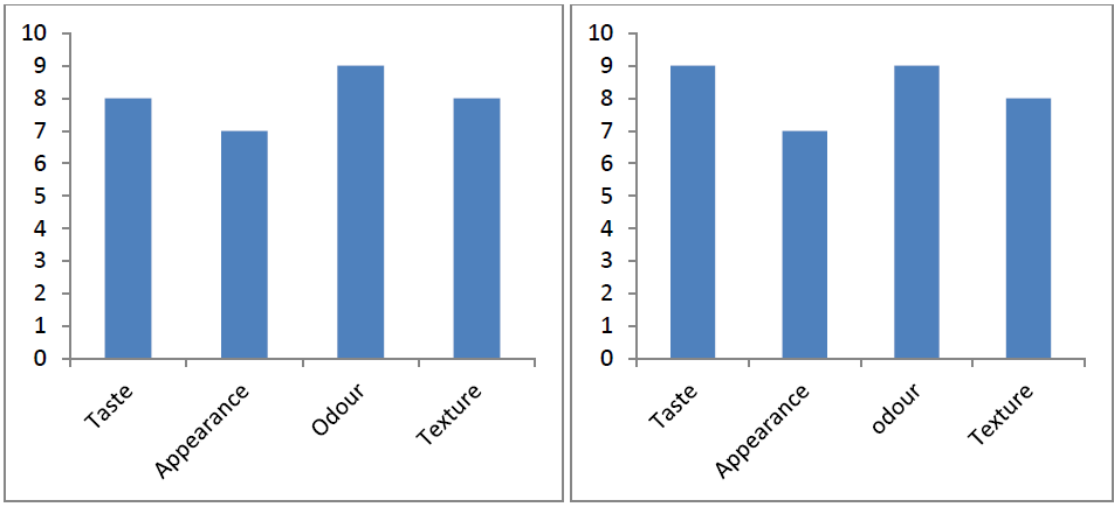

Fig 3: Graphs representing sensory evaluation for the formulation 1 and 2 respectively.

4. Conclusion

Coix lachryma-jobi L. is a widely grown and studied crop in China. However, in India it is grown only in Andhra Pradesh and needs to be utilized more. It is one of the underutilized grains of India and can find applications in food industry in a broader prospect if given attention. It is healthy and above all very good in protecting cancer, if included in the daily diet. The study proves it to be a potent antioxidant with a considerable amount of phytochemicals, which widens its way more to be used as a base in the processed products that are consumed on a daily basis.

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