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FORMULATION OF HERBAL POWDER SACHET USING *DENDROBIUM CHRYSOTOXUM* FOR IMMUNOMODULATORY ACTIVITY IN HIV PATIENTS

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Abstract: *Dendrobium chrysotoxum* is a type of orchid in the family of *orchidacae* with wide range of medicinal properties.These species widely used in traditional chinese medicine.It have antipyretic,antiinflammatory and immunomodulatory activities. Current study was carried out to formulate a neutraceutical sachet of *Dendrobium chrysotoxum* plant for HIV patient.. HIV patients lacks immunity, *Dendrobium chrysotoxum* help them to increase the immunity by stimulating splenocyte proliferation.The immunomodulatory activity of dried powder was tested and it was found to have potent immunomodulatory activity.Qualitative and quantitative studies are done to assure the quality and quantity of active ingredients. The powder sachet is formulated by adding saccharin as sweetening agent and sodium benzoate as preservative.Evaluation and standardization parameters of formulation were done.The product is prepared under nutraceutical guidelines. The formulated sachet can be given as health drink for HIV patients.

***Index Terms* - *Dendrobium chrysotoxum* , Neutraceutical,Immunomodulatory,HIV patient**

INTRODUCTION

Orchidaceae is one of the largest families among angiosperm. Orchids are cultivated for beautiful flowers and ornamental purposes. According to one estimate the orchidaceae family includes 800 genere and 25000 species. Orchids thrive in regions from sea level to atleast 4600 meters in elevation. The greatest number of orchids species is found usually on mountainsides. Arunachal Pradesh has been termed as 'Orchid Paradise of India' because highest number of orchid species (around 622 species) are founded.[1,2,3]

Phytochemically some orchids have been reported to contain alkaloids, tripenoids, flavonoids, and stilbenoids. Some plants like *Dendrobium crumenative, Eulophia campestris, Orchis latifolia, Vanda roxburghii* and *Vanda tessallata* have been documented for their medicinal value. Ayurvedic formulations like chyvanprasha and for plants like Riddhi, Jivaka, Vriddhi have been discussed as possible members of family Orchidaceae.

The medicinal property of orchids where first discovered by the chinese. The chinese continues to use orchids for medicinal purposes until today.

Dendrobium is the second largest genus.*Dendrobium* name coming from Greek orgin :"dendros" ,tree and "bios", life.It is used in traditional chinese medicine as a tonic to improve the digestion and promoting the production of body fluid. *Dendrobium* namely ,*D. chrysotoxum* ,*D. chrysanthum, D.officinale, D.nobile,*

and D..candidum etc...These are the sources of astringent, analgesic, antipyretic , immunomodulatory and anti-inflammatory substances.

The *Dendrobium* species are used to cure different types of diseases like skin diseases, sexual weakness, general weakness etc. The plant's tubers are commonly used to treat maximum number of diseases and then whole herb,rhizomes,stems and leaves.The medication prepared by this species are taken orally or administered externally. The formulation are decoction, infusion, tea, poultice and paste.[4,5,6]

AIDS stands for acquired immunodeficiency syndrome a pattern of devastating infections caused by HIV, which attacks and destroys certain white blood cells that are essential for immunity.AIDS is a fatal disease. Research continuous on possible vaccines,ultimately a cure. However prevention of transmission remains the only method of control.

Dendrobium chrysotoxum is also known as golden bow dendrobium. It is a epiphytic,clump forming orchid,up to 30 cm tall;with fleshy green pseudobulbs carrying two to three dark green, lance shaped leaves,10-18cm long. The native place is Arunachal Pradesh to China and indo-china,. It is a pseudobulb epiphyte and grows primarily in the wet tropical biome.

Dendrobium chrysotoxum is a major variety in dendrobiums.it have antipyretic, anti-inflammatory and immunomodulatory activities.it consist DCP W4 , a polysaccharides which have immunomodulatory activity that promote splenocyte proliferation.it is rich with vitamin c and fibers.This powder helps to increase immunity in HIV patients. So a powder sachet is prepared with this powder..[7,8,9]

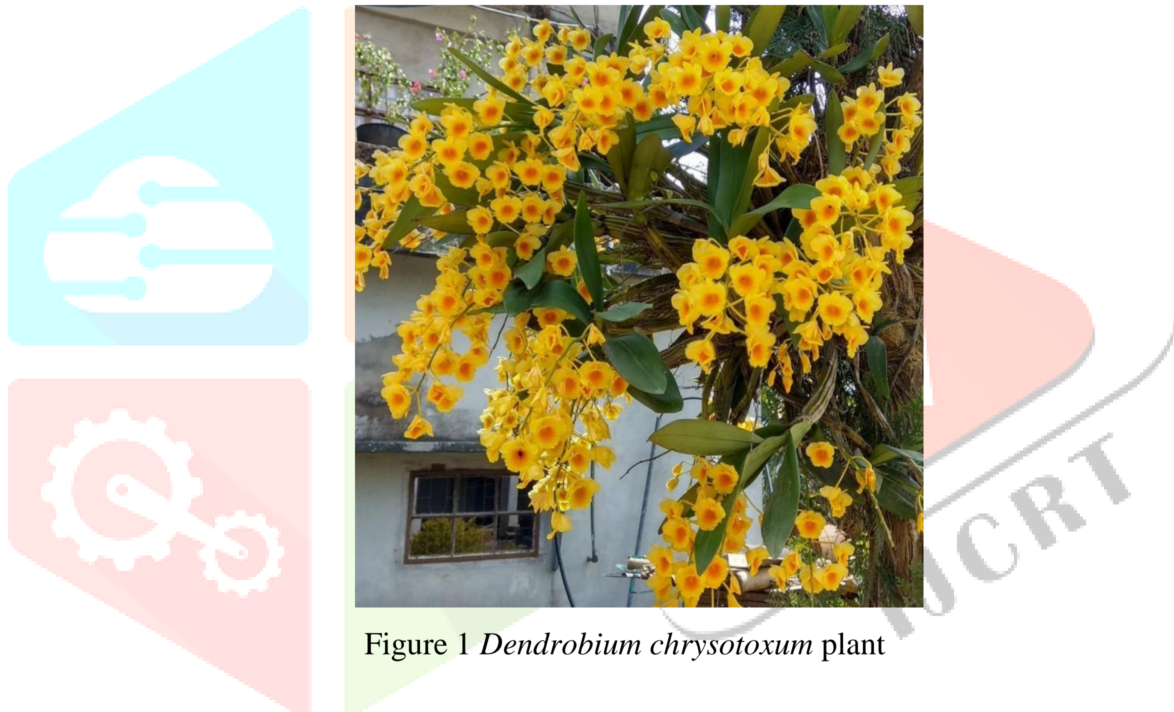


Figure 1 *Dendrobium chrysotoxum* plant

RESEARCH METHODOLOGY

Plant collection and drying

The plant were collected from Government nursery and the plant was authenticated by Dr.M.Bheemalingappa Scientist B, Forest Botany Department, KSCSTE- Kerala Forest Research Institute Peechi.

The leaves, pseudobulbs,and stems of samples were thoroughly washed with clean water and chopped into small pieces. Later it shade dried daily to facilitate rapid dehydration. properly dried to avoid deterioration. The dried plant is powdered well and sieved in sieve no:80 , stored in a desiccator .[10,16]

Figure 2 *Dendrobium chrysotoxum* collected plant

<u>Qualitative tests</u>
Determination of various ash values
Total ash

Total ash generally consists of phosphates, carbonates, silica and silicates which include both physiological ash and non physiological ash, eg, sand and oil. 2 g m of air dried powdered drug was accurately weighed and taken in a silica crucible and incinerated at 450°C to free from carbon. Then the crucible was cooled and weighed. The percentage of total ash was evaluated with reference to the dried drug.

Water-soluble ash

Water soluble ash is that part of the total ash portion which was soluble in water. Then total ash obtained was boiled for 5 minutes with 25 ml of water and insoluble material was collected in an ash less filter paper, incinerated at 450°C, subtracted the weight of the insoluble substance from the weight of the ash and computed the rate of water soluble ash amid reference to their dried drug.

Acid-insoluble ash

The acid insoluble ash is calculated by treating the total ash with dilute HCL and weighing the residue. This limits indicates the contamination with siliceous materials like sand by comparison with total ash value of the same sample differentiation can be made between contaminating material and in the natural ash of the drug. The total ash was obtained by boiling with 25 ml of 2 N HCL for 5 min while the insoluble matter was collected in an ash less filter paper and washed with boiled water followed by ignition and cooling in desiccator and finally weighing. The proportion of acid insoluble ash with reference to the dried drug was evaluated

.

Sulphated ash

1 gm of air dried powder was treated with dilute sulphuric acid before ignition in a tared silica crucible to a constant weight. The ash obtained was weighed. The percentage of sulphated ash was intended with reference to the dried drug.

Loss on drying

About 3gm of the powdered crude drug was accurately weighed in a tarred dish and dried in an oven at 100-105°C. It was then cooled in a desiccators and weighed. The loss on drying was calculated with reference to the amount of the dried powder taken
Loss on drying = (weight of empty desiccators + sample weight – weight after drying)/sample weight

Determination of swelling index

Swelling property of medicinal plant shows specific therapeutic utility e g: gums, pectin, or hemicellulose. 1 gm of plant material was shaked thoroughly in every 10 min for 1 hr and finally allowed stand for 3 hrs at room temperature. Measured the volume in ml occupied by plant material and calculated the mean value of individual determination, related to one gm of crude plant material.

Determination of foaming index

The foam forming capability of plant material and their extract is measured in term of foaming index. 1 gm of powdered root was accurately weighed and transferred in to a 500 ml conical flasks containing 100 ml water and boiled for 30 min, cooled and filtered into 100 ml volumetric flask and volume was made up with water. The decoction was poured into ten stoppered test tubes in consecutive part of 1 ml; 2 ml; etc up to ten ml and adjusted the volume of each test tube with water to 10 ml and shaken them in lengthwise motion for 15 sec. Allowed to stand for 15 min and measured the height of the foam. The results were assessed as follows:
If height of foam in every tube was less than 1 cm the foaming index was considered less than 100.

If height of the froth was heigher than that of 1 cm in every tube the foaming index was over than 1000. In such case repetitions was done by using a new series of dilutions of decoction in order to obtain the result.

If height of foam in any test tube was 1 cm, and volume of the crude plant material decoction in that tube (a) was used to determine the index.

Formula used for calculation of foaming index = a/1000
A = Volume of decoction that was used for preparing the dilution in tube where foaming height was 1 cm measured.

Phytochemical screening
It is the screening of revealing the presence of alkaloids, saponins ,tannins ,coumarins, terpenoids etc.[16]

Test for alkaloids

For the qualitative test for alkaloid, 5 alkaloid detecting reagents are used. These were –Drangendroff's reagent,hager's reagent,mayers reagent,wagner's reagent,tannic acid reagents are used.
5gm fresh finely chopped and pasted plant material was mixed up to moisten with 10ml 2% HCL and heated in waterbath for 60°C for one hour . After cooling the extract was filtered through Whatman No:1 filter paper. Two drops of extract was placed on microscopic groove slide with one drop of alkaloid detecting agent. The relative abundance of precipitate , if any formed in the plant extract with the reagent was considered as an index of quality of the presence of alkaloid and was expressedby'+','++', '+++' signs which mean slight, moderate, substantial to a heavy amount respectively. No precipitate was indicated by '–' (negative sign) and stood for the absence of alkaloid in the plant extract.

Test for phlobatannins

Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid was taken as evidence.

Test for flavonoids

A portion of the crude powdered plant sample was heated with 10ml of ethyl acetate over a steam bath for 3 min. the mixture was filtered and 4 ml of filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration was observed indicating a positive test for flavonoids.

Test for saponins

About 2gm of crude powder was boiled with 20ml of distilled water in a water bath and filtered. 10 ml of filtrate was mixed with 5 ml of distilled water and shaked well for a persistent froth. This froth indicate presence of saponins.

Test for tannins

About 0.5gm of crude powdered samples are boiled in 10 ml of distilled water in a test tube and filtered . A few drops of ferric chloride reagent added to the filtrate. A blue black precipitate was formed when the tannins are present.

Test for terpenoids

0.5gm of crude powder was dissolved was dissolved in 5ml methanol.5nl of the extract was treated with 2ml of chloroform in a test tube. 3mlo of concentrated sulphuric acid is carefully added by the sides of the test tube to form a layer. An interface with a reddish brown coloration formed if terpenoid is present

.

Test for steroids

0.5gm of crude powder was dissolved in 5ml of methanol 1ml of the extract was dissolved in10ml of chloroform and equal volume of concentrated sulphuric acid was added by the side of the test tube. The upper layer turns layer turns red and sulphuric acid layer showed yellow with green flourescenece. This indicated the presence of steroids.

Test for glycosides

0.5gm of crude powder was dissolved in 5ml of methanol.10ml of 50% HCL was added to 2ml of methanolic extract in the test tube . Then it was heated in a boiling water bath for 30minutes. 5ml of Fehling solution was added to the mixture and the mixture was boiled for 5 minutes. A brick red precipitate was taken as evidence for the presence of glycosides.

Test for anthraquinone

2ml of solution was added with Magnesium acetate. Formation of pink color indicates the presence of Anthraquinones.

Test for quinine

1ml of xtract, 1ml of concentrated sulphuric acid was added and was allowed to and for some time to develop color. Development of red color indicate presence of quinine.

Test for coumarins

1 ml of extract , 1ml of 10% Sodium hydroxide was added ans was allowed to stand for some time development of yellow colour shows presence of coumarin.

Quantitative analysis

Quantitative analysis is the determination of the absolute or relative abundance of one, several or all particular substance present in sample.[11,12,13,14]

Total alkaloids content:

5 g of the plant sample is prepared in a beaker and 200ml of 10% in is added to the plant sample. The mixture is covered and allowed to stand for 4 hours.The mixture then filtered and the extract is allowed to become concentrated in a water bath till it reaches of the original volume. Concentrated is added until the precipitation is complete.The whole solution is allowed to settle and the precipitate is collected and washed with dilute and then filtered.The residue is alkaloid,which is then dried and weighed.

Total tannins content:

The tannins were determined by Folin-Ciocalteu method. About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of FolinCiocalteu phenol reagent, 1 mi of 35% sodium carbonate solution and dilute to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. a set of reference standard solutions of tannic acid (20, 40,

60, 80, 100 µg/ ml) were prepared in the same manner as described earlier. Absorbance for test and standard solutions were measured against the blank at 700 nm with an UV/ Visible spectrophotometer

Total flavonoid content:

10 g of plant sample is repeatedly extracted with 100 ml of 80% aqueous methanol at room temperature.The whole solution is then filtered through filter paper and the filtrate is later on transferred into a water nath and solution is evaporated into dryness.The sample is weighed until a constant weight.

Total carbohydrate content:

Take 7 clean, dry test tubes. Pipette out standard sugar solution in the range of 0 to 3 mL in different test tubes and make up the volume of all test tubes to 3 mL with distilled water concentrations ranging from 0 to 750 mg. Add 1 mL DNS reagent to all the test tubes and mix plug the test tube with cotton or marble and keep the test tube in a boiling water bath for 5 minute. Take the tubes and cool to room temperature. Read extinction at 540 mm against the blank. Please note that all the tubes must be cooled to room temperature before reading.
Prepare standard curves of the sugars provided and use them to estimate the concentration of the unknowns provided.

Total fiber content:

2g of powdered drug extracted with diethyl ether and added 200ml of boiling dilute Sulphuric acid (1.25%) to the ether exhausted marc in a 500ml flask. The mixture was refluxed for 30min, filtered through filter paper and the residue was washed with boiling water until the effluent washing was acid free. The residue was rinsed in 200ml of boiling sodium hydroxide solution (1.25%) and was further refluxed for 30min finally it was filtered through ash less filter paper. The residue was washed with boiling water until the last washing was neutral. It was then dried at 110°C to constant weight and then ignited to constant weight. The ash was cooled in dessicator, weighed and calculated as follows

Percentage of crude fiber = weight of the ash obtained/weight of the drug sample×100

Total protein content:

First take different aliquots of standard protein solution ranging from 0.1 to 1.0 ml. Then take suitable aliquots of undiluted and diluted unknown sample and make up final volume to 1.0 ml with distilled water. After that, add 5.0 ml of alkaline copper reagent in all the test tubes. Mix thoroughly and incubate at room temperature for 15 minutes. At the end, prepare final mixture by adding 0.5 ml Folin's Ciocalteau reagent in all the test tubes and mix well. Incubate it at room temperature for 30 minutes. Measure the intensity of blue color colorimetrically at 750 nm. Detect concentration of protein in unknown sample by drawing the standard graph**.**

In vitro immunomodulatory activity

Immunomodulation through natural substances may considered as an alternative for the prevention and cure of neoplastic diseases. There is a growing evidence such as polysaccharides from natural plants can significantly enhance the immune system. They are regarded as promising immunomodulatory agents which are relatively non toxic and have no significant side effects. *Dendrobium chrysotoxum* act as a health promoting and therapeutic tonic. The polysaccharides from *D.chrysotoxum* are considered to play an important role in medical effects. Through enzyme-assisted extraction of DCP and at investigating the physiochemical characteristics and functional properties of DCP-E obtained from enzyme-assisted extraction and DCP-H was obtained by hot water extraction.
Materials and Methods
Method: MTT Assay

Cell line used:

Specific pathogen free (SPF) male BALB/c mice, 8 weeks old. Animals were maintained in a temperature controlled environment (22±2°C) with a 12h light/dark cycle and with free access to water and rodent chow. RAW264.7 macrophages were cultured in Dulbecco's modified Eagle's medium and YAC-1 cells were cultured in RPMI-1640 medium both supplemented with 10% fetal bovine serum, 100IU/ml penicillin, and 100μg/ml streptomycin in a 95% humidified atmosphere containing 5% carbon dioxide at 37°C.

Cell treatment (Splenocyte proliferation assay) procedure:

Spleen from BALB/c was removed for preparation of splenocytes. The cells were freed of red blood cells by treatment with lysis buffer (0.15M NH4Cl, 0.01M KHCO3, and disodium salt of ethylene diamine tetraaceticacid (NA2EDTA), pH 7.4).

To remove adherent cells such as macrophages, splenocytes were incubated for 2hr in pertri dishes. The suspended cell populations were collected and used as the Splenocyte populations. DCP- H and DCP-E were dissolved in phosphate buffer saline (PBS) for experiments. Mouse splenocytes were adjusted to 2×10^6 cells/ml in RPMI-1640 medium. Then the cell suspension was distributed (100μl per well) on 96 well plates and cultured and examined he DCP-H and DCP-E with differences in Effect of pH and Effect of temperature.

Splenocytic proliferation activity was tested by MTT assay.

Splenocytic proliferation index =ODexperimental / ODcontrol

MTT Assay:

Principle

The colorimetric assay is based on the capacity of a mitochondrial enzyme in a living cells, succinate dehydrogenase, cleaves the tetrazolium ring, converting the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) into a insoluble purple formazan product which is measured spectrophotometrically. Only viable cells with active mitochondria reduces the MTT, the amount of formazan produced is directly proportional to the number of viable cells.

Procedure:

After 48hr of incubation,15μl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hr. The medium with MTT was then flicked off and the formed formazan crystals were solubilized and then measured the absorbance at 570nm using 96 well plate counter.

%Cell inhibition = 100 -Abs (sample) /Abs (control) ×100

RESULTS AND DISCUSSION

Drying

The herb was shade dried up to 25 days then the herb was crushed into fine powder.
Total 50 g of herbal powder was obtained.

Qualitative test

Table 1: Determination of ash value

Sl no	extracts	Total ash value	Acid insoluble ash	Water insoluble ash	Sulphated ash
1	Plant	14.8	0.9	3.1	7.2

Table2:values obtained on loss on drying,swelling index,foaming index

Sl no	parameter	Observation in plant
1	Loss on drying	16.23
2	Swelling index	No significant result
3	Foaming index	No significant result

Phytochemical screening

Table 3:phytochemical screening

Sl no	Secondary metabolites	Results (% of coloration)
1	Alkaloids	+++
2	Glycosides	+++
3	Flavanoids	+++
4	Phlobotannins	-
5	Saponins	+
6	Tannins	++
7	Terpenoids	+++
8	Steroids	+++
9	Anthroquinone	-
10	Quinine	+++
11	coumarin	+++

Quantitative analysis

Total alkaloid content:

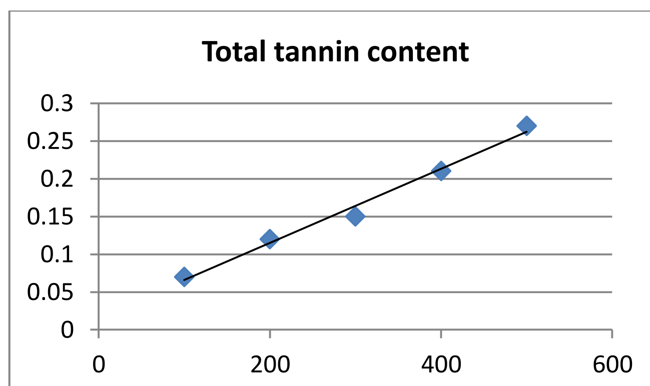
Weight of the residue=3.01mg

Total tannin content:

Table 4:determination of total tannin content

Concentration(µg/ml)	Absorbance (nm)
100	0.07
200	0.12
300	0.15
400	0.21
500	0.27
Sample	0.2

Graph 1:total tannin content



Concentration of sample=372.532µg/ml

Total flavonoid content:

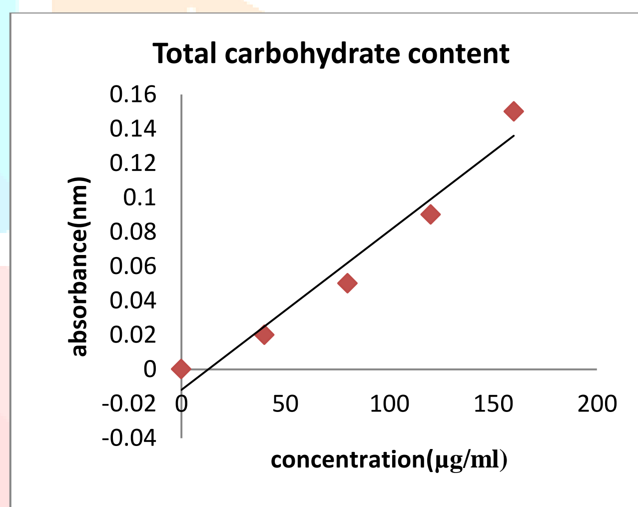
Weight of the residue=0.498mg

Total carbohydrate content

Table 5:determination of total carbohydrate content

Concentration(µg/ml)	Absorbance(nm)
40	0.02
80	0.05
120	0.09
160	0.15
200	0.19
Sample	0.12

Graph 2:total carbohydrate content



Concentration of sample=136.229µg/ml

Total fiber content

Percentage crude fiber = $\frac{weight\ of\ the\ ash\ obtained}{weight\ of\ the\ drug\ sample}$×100

weight of the ash obtained=0.1652g

weight of the drug sample=2g

Percentage crude fiber = $\frac{0.1652}{2}$×100

=8.26%

Total protein content

Table 6 :total protein content

concentration(µg/ml)	Absorbance(nm)
1	0.04
2	0.09
5	0.21
10	0.4

Concentration of sample=6.149µg/ml

Graph 3:total protein content

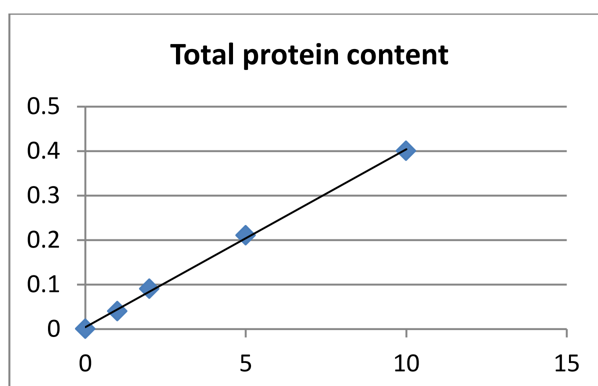


Table 7:overall values of alkaloid,protein,flavanoid etc..

Total alkaloid content	0.0031g
Total tannin content	0.372g/l
Total flavonoid content	0.000498g
Total carbohydrate content	0.136g/l
Total Protein content	0.006149g/l
Total Fibre content	8.26%

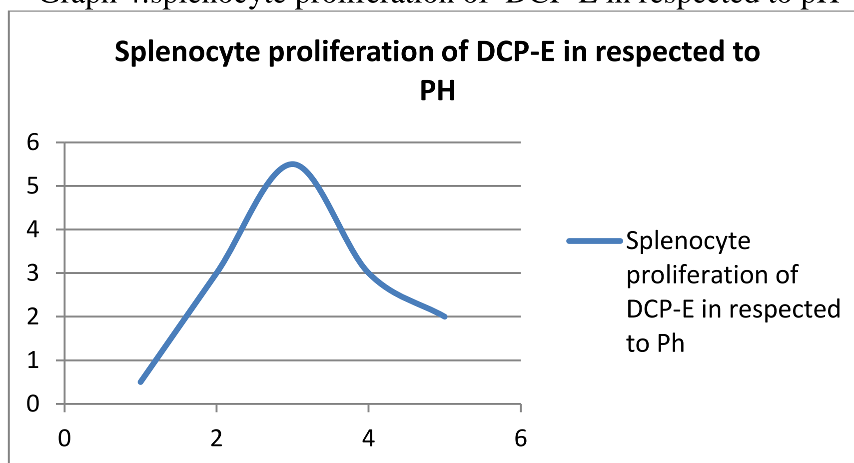
Invitro immunomodulatory assay

The *in vitro* immunomodulatory activity of *Dendrobium chrysotoxum* were given below as, The spleen of the body's largest immune, organ cample of producing large number of lymphocytes. All the polysaccharides at low concentrations showed no effect on the Splenocyte proliferation compared to the control.

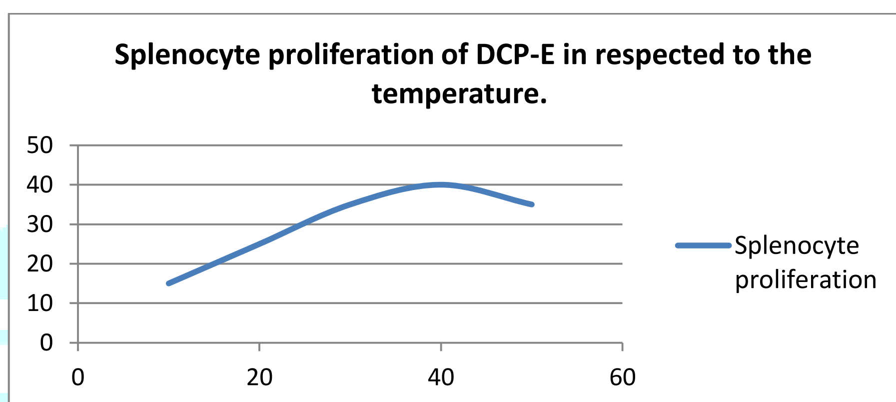
Due to the effect of pH values, the SPI of DCP-E increased with an increasing pH values from 2 to 6 then decreased when pH exceeds to 6.

Due to the effect of temperature, the SPI of DCP-E increased postively from 10 to 50.

Graph 4:splenocyte proliferation of DCP-E in respected to pH



Graph 5:Splenocyte proliferation of DCP-E respected to temperature



Among the polysaccharides, the Splenocyte proliferation index of DCP-E is greater than that of DCP-H . The optimum conditions where the pH values 5.5, the temperature is 40°C.
In addition to that ,compared to DCP-H the DCP-E have increased purity, decreased molecular weight, relative velocity as well as changed monosaccharide composition.

Based on the study mentioned above ,we should conclude that *D.chrysotoxum* have significant *in vitro* immunomodulatory activity. Both DCP-H and DCP-E may potentially served as immunomodulators.

Formulation of herbal powder sachet

The collected plant is washed well to remove the foreign matters present in it and dried well in shade . Drying in direct sunlight may cause deterioration of the product. The product is powdered well and sieved in sieve no:80 and stored in room temperature.
The powder is mixed with sodium benzoate as preservative and saccharin as sweetening agent. and packed as sachets.

Ingredients

Orchid powder
Sodium benzoate
Saccharin

Table 8: amount of carbohydrate,protein,fiber in 1g of sample

constituents	Amount in 1g
Carbohydrate	68.114g/l
Protein	3.07g/l
Fiber	4.13g/kg

Table 9: Ingredients and their weight to be taken

INGREDIENTS	OFFICIAL FORMULA	WEIGHT TO BE TAKEN(4gm)
Dendrobium chrysotoxum powder	20g	4g
Sodium benzoate	20mg	0.004g
Saccharin	25mg	0.00425g

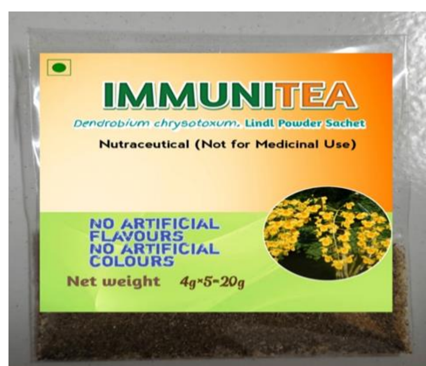


Figure 3 Herbal powder sachet

Report

Powder sachets containing *Dendrobium chrysotoxum* was successfully developed.it have good immunomodulatory activity because of the polysaccharide DCP W4. And it increases the immunity in HIV patients . It can be consumed as a nutraceutical to boost the immunity.

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