FORMULATION DEVELOPMENT, EVALUATION AND MTT ASSAY OF HERBAL VITAMIN C POWDER.

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Abstract: The present work was aimed at formulation development, evaluation and MTT assay of Herbal Vitamin C Powder. The formulation was prepared by mixing Acerola Extract, Rosehip Extract, Moringa Extract, Amla Powder, Bilberry Extract, Glycine, L- lysine, L- proline, Elaichi Powder, Rock salt, Spirulina Powder and excipients such as Lemon flavor. Initially, the pre-formulation evaluation of each separate ingredient was done by evaluating its Organoleptic characters, Bulk density, Tap density, Hausner Ratio, Moisture Content, pH, and Solid-state stability study. The formulation was prepared after the pre-formulation study was completed and then evaluated for powder characters and MTT assay. Powder evaluation was performed using various physiochemical and microbiological parameters and the cytotoxicity of the formulation was performed by an MTT assay on Human embryonic kidney cells (HEK). From the above study, we can conclude that the stable polyherbal formulation of Vitamin C was prepared and evaluated and did not exhibit cytotoxicity at the daily dose limit.

Keywords: Vitamin C, MTT assay, Cell viability assay, Human embryonic kidney cells

Introduction:

The new single chemical entity is responsible for the medicine's significant therapeutic activity in modern pharmacology and drug development. Still, Ayurveda formulations are founded on different standards: using a single herb and multiple herbs, known as polyherbal formulation. Polypharmacy or polyherbalism refers to the impact of combining several medicinal plants to increase the efficacy of a preparation.[1] Historically, the classic text of Ayurveda, "Sarangdhar Samhita," has emphasized the concept of polyherbalism.[2] Although the bioactive components of individual herbs in many formulations have been well-known, they generally exist in small proportion. They are mostly not sufficient enough to attain the desired therapeutic efficacy. Scientific studies have discovered that when mixed, these herbs of varied effectiveness may yield better results concerning the single or sum of their single effect. This occurrence gives rise to positive herb interaction known as synergism which confers some benefits of the polyherbal formulation. It is evident that with a single multi-constituent formulation, better therapeutic outcomes can be attained.[1] The term "nutraceuticals" can be explained as the food items as a whole or a part which possesses some nutritional value along with medicinal properties.[3] These findings have triggered a series of studies in the nutraceuticals field.[4] There is a controversy over a specific definition and set of regulations to define nutraceutical compounds.[5] However, nutraceutical compounds are health-enhancing products that improve the mental and physical activities of the body. They are commercialized to minimize the risk factors of various diseases. Nutraceutical products are simply a hybrid between drug and food. On the other hand, this terminology is a broader term that includes minerals, vitamins, amino acids, botanicals, or herbs. Therefore, both dietary supplements and fortified foods can be classified as nutraceuticals. The terminology of nutraceutical was defined by the foundation for innovation in medicine in (New York, USA) in 1989. Defelice's definition in 1995 was: "A food or parts of food that provide

medical or health benefits, including the prevention and/or treatment of disease.[5] Nutraceuticals term originated from two terminologies: "nutrition" and "pharmaceutical". [6]

Vitamin C (ascorbate) is an essential water-soluble micronutrient in humans and is obtained through the diet, primarily from fruits and vegetables.[7] Vitamin C is necessary for the development and maintenance of connective tissues. It plays a vital role in bone formation, wound healing, and the care of healthy gums. It helps synthesize and metabolize tyrosine, folic acid and tryptophan and hydroxylation of glycine, proline, and lysine carnitine.[8] It is a cofactor for collagen synthesis and a primary antioxidant and is rapidly consumed post-wounding. Vitamin C could promote wound healing by altering the inflammatory, proliferative and remodelling phases. Vitamin C protects the immune system, reduces the severity of allergic reactions, and helps to fight off infections.[9] Humans cannot synthesize ascorbic acid due to the lack of gulonolactone oxidase enzyme. Hence, ascorbic acid has to be supplemented mainly through fruits and vegetables. It is present in oranges, lemons, grapefruit, watermelon, papaya, strawberries, mango, pineapple, raspberries and cherries. It is also found in green leafy vegetables, tomatoes, broccoli, green and red peppers, cauliflower and cabbage.[10]Vitamin C is a cofactor in the hydroxylation of proline and lysine residues in procollagen, which is vital for the strength and stability of collagen fibers. In addition, ascorbic acid enhances neutrophil function and acts as an antioxidant.[5]Systemic administration of vitamin C plays a vital role in gingival fibroblast proliferation and functions.[6]According to ICMR guidelines, adults' daily allowance (RDA) for ascorbic acid ranges between 70–90 mg daily.[7]The polyherbal formulation comprises Acerola (Malpighia emarginate DC.), known as Barbados cherry or West Indian cherry, which belongs to the Malpighiaceae family. The fruit is known to be one of the world's rich natural sources of ascorbic acid. Apart from containing an exorbitant amount of ascorbic acid, the fruit

also contains several phytonutrients like carotenoids, phenolics, flavonoids, and anthocyanins and possesses numerous bio-functionalproperties.[8] The rosehip is a repository of flavonoids, pectin, vitamins A, B complex, C and E, also minerals like Ca, Fe, Se and Mn. Trace amounts of Mg, K, S and Si have also been discovered.[9] Moringa leaves are an essential source of several nutrients. One hundred grams of dried leaves contain 27.1 g protein, 16.3 mg vitamin A, 17.3 mg vitamin C, 2.0 g calcium, 1.3 g potassium, and 28.2 mg iron, in addition to 19.2 g dietary fiber and several other nutrients.[10] Along with the active vitamin C ingredients glycine, L-proline and L-lysine were added, which metabolize by the vitamin C easily and get maximum benefits.[11]Also, some taste improvements agents such as Elaichi and Rock salt were added to the formulation. The supplementation of spirulina as a potent alternative source of iron and folic acid. Ascorbic acid facilitates iron absorption by forming a chelate with ferric iron at an acid pH that remains soluble at the alkaline pH of the duodenum.[12]

In the present study, the biological effects of vitamin C on cells were investigated in vitro by using the MTT assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test was used to explore the cell viability in the MTT assay. The MTT colorimetric assay was conducted in a 96-well plate format. The cells may require preincubation in the wells before adding the test drug. The preincubation times may vary from 0-24 hrs. according to the cell line properties. Cells are usually exposed to the drug for 24-96 hrs. depending on the drug activity. MTT solution is then added to the treated cells, where the yellow MTT is reduced to purple formazan by various mitochondrial and cytosolic enzymes that are operational in viable cells. The MTT molecule is not reduced by dead cells, red blood cells (metabolically inactive cells), spleen cells (resting cells) and Stimulated lymphocytes (activated cells). After 3-4 hrs. of incubation with MTT, the formazan absorbance at 550 nm is directly proportional to the number of cells in a range of 200-

50,000 cells per well, and thus very small amounts of cells can be detected. The absorbance indicates the number of viable cells remaining after treatment with the drug and is compared to the absorbance of control cells not exposed to the drug.[13].

Material and methods:

Materials:

Methanol, sulfuric acid, acetic acid, toluene, ethyl acetate, formic acid (Merck made), alpha naphthol, ferric chloride, potassium permanganate, indigo carmine (Loba Chemie made) and polysorbate 80, etc. which are of AR grade and procured from Vijay chemicals, Pune. For microbiological evaluation, chemicals like Soyabean casein digest medium, Gram Negative broth, Rappaport Vassiliadis Salmonella Enrichment Broth, Soyabean casein digest agar, Xylose lysine deoxycholate agar, Bismuth sulphate agar, Cetrimide agar, MacConkey broth & agar, sabouraud dextrose broth & agar (HIMEDIA made) which are of LR grade and procured from Vijay chemicals, Pune. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DMEM media (Gibco), and Fetal bovine serum (Gibco) were procured from genexindia Bioscience.

Instruments:

1. Weighing Balance (Master)
2. Hot Air Oven (Bio-Techniques India)
3. pH Meter (Global)
4. Muffle Furnace (Bio-Techniques India)
5. Mechanical Shaker (Bio-Techniques India)
6. Laminar airflow (Bio-Techniques India)
7. Bacteriological Incubator (Bio-Techniques India)
8. CO_2 incubator
9. Automated microplate reader

Methods:

Pre-formulation studies were done by performing color, odor, taste, bulk density, tap density, Hausner ratio, carr index, angle of repose and solid-state stability. Organoleptic Properties of the formulation like color, odor, taste and powder characteristics like bulk density, tap density, Hausner ratio, carr index and angle of repose, were determined. Physiochemical stability was checked by performing loss on drying, pH, total ash, acid insoluble ash value, water-soluble extractive value, alcohol-soluble extractive value and also the presence of phytochemical constituents like tannin, phenols, flavonoids and carbohydrates was checked.

The microbial evaluation was done by performing the total aerobic bacterial count, total aerobic fungal count and specific pathogen test for Escherichia Coli**,** Staphylococcus Aureus, Salmonella Species and Pseudomonas aeruginosa. The nutritional values were also estimated and the MTT assay was performed to assess the cytotoxicity of the formulation.

A. Pre-formulation Study

Pre-formulation is a phase of the research and development process in which a new medicinal molecule's physical, chemical, and mechanical properties are studied individually and in combination with excipients to generate a stable, safe, and effective bioavailable dosage form.

1. Organoleptic Properties:

Color: Observation was done on bright background with light using drug powder. It was carefully observed by the naked eye.

Odor: Before smelling coffee, beans were used to remove all previous odors. Powders took in between the thumb and 1st finger and smelled it.

Taste: Powders are taken, tasted on the tongue, and examined for the type of taste.

2. Powder characteristics:

Bulk Density:

5 gm of the powdered drug was weighed in the digital balanced weighing machine. These powders were added to the dried 25 ml graduated cylinder. The volume of the cylinder was noted.[15]

Bulk Density = M/ V

Where, M = mass of powder

V = Volume of powder

Determination of tap density:

5 gm of the powdered drug was weighed in the digital balanced weighing machine. These powders were added to the dried 25 ml graduated cylinder. After measuring the initial volume of a cylinder, it was mechanically tapped using a tap density apparatus of BIO TECHNICS INDIA, BTI-08. The final volume was noted.[15]

Tapped density = M/ Vt

Where, M = Mass of powder

Vt = Minimum volume occupied after tapping

Hausner Ratio:

Hausner ratio (HR) indicates the powder's flow characteristics and flowability. The ratio between the bulk densities of compacted and loosely poured powder is called Hausner.[16]

Hausner Ratio = ρ tapped/ ρ bulk

Where, ρ = Density

Carr Index:

Carr index gives an idea indirectly about the flow behavior of a powder. Carr index (Ci) is determined using Hr values as given in Eq. [16]

Hr = 100 - (100/ Hr)

Angle of repose:

In the fixed funnel method, the granular materials are poured from a funnel at a certain height onto a selected base with known roughness properties. The funnel is either fixed or raised slowly while the conical shape of the material heap is forming to minimize the effect of the falling particles. The pouring of the material is stopped when the heap reaches a predetermined height or width. Then, the angle of repose is measured by the inverse tangent (arctan) rule, at which the average radius of the formed conical shape and the maximum height of the heaped material is measured. Then the angle of repose is determined as the arctan of the maximum height.[17]

The following formula can calculate the angle of repose:

$$\tan \theta = h/r$$

So,

$$\theta = \tan^{-1} h/r$$

Where,

θ = Angle of repose

h = height of pile of powder(cm)

r = radius (cm)

Moisture Content:

In the dried petri dish 1.5 gm weighed powder was taken and placed in an oven at 105– 110^0c. After drying and cooling in a desiccator, it was weighed in a digital balanced weighing machine. After drying, the weight was reported and the drying loss was measured.[17]

pH:

In a digital balanced weighing machine, 1 gm of powder was weighed and mixed with 20 ml of distilled water. pH of this solution was calculated using a digital pH meter.[17]

Solid State stability study:

Solid-state reactions are much slower and more difficult to interpret than solution-state reactions, due to a reduced no. of molecular contacts between drug and excipient molecules and to the occurrence of multiple-phase reactions.

A small mixture of drug and excipient was prepared. The mixture was then placed in the vial. A rubber closure was placed on the vial and the stopper was dipped in the melted Carnauba wax to seal. Then the vials are kept for 1-3 weeks for specified storage conditions. The sample was physically observed for the following.[17]

1) Caking
2) Liquefaction
3) Discoloration

4) Odor
5) Gel formation

B. Formulation Evaluation:

1. Organoleptic Properties:

Color: Observation was done on bright background with light using drug powder. The naked eye carefully observed it.

Odor: Before smelling coffee, beans were used to remove all previous odors. Both Powders took in between the thumb and 1st finger and smelled it.

Taste: Both Powders took and tasted on the tongue and examined for the type of taste.[14]

2. Powder characteristics:

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Tapped density = M/ Vt

Where, M = Mass of powder

Vt = Minimum volume occupied after tapping

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Hausner ratio (HR) indicates the powder's flow characteristics and flowability. The ratio between the bulk densities of compacted and loosely poured powder is called Hausner.[16]

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In the fixed funnel method, the granular materials are poured from a funnel at a certain height onto a selected base with known roughness properties. The funnel is either fixed or raised slowly while the conical shape of the material heap is forming to minimize the effect of the falling particles. The pouring of the material is stopped when the heap reaches a predetermined height or width. Then, the angle of repose is measured by the inverse tangent (arctan) rule, at which the average radius of the formed conical shape and the maximum height of the heaped material is measured. Then the angle of repose is determined as the arctan of the maximum height.[17]

The following formula can calculate the angle of repose:

$$\tan \theta = h/r$$

So,

$$\theta = \tan^{-1} h/r$$

Where,

θ = Angle of repose

h = height of the pile of powder (cm)

r = radius (cm)

3. Physiochemical parameters:

Determination of loss on drying:

In a dried petri dish, 1.5 gm weighed vitamin C powder was taken and placed in an oven at 105– 1100 c. After drying and cooling in a desiccator, it was weighed in a digital balanced weighing machine. After drying, the weight was reported, and the drying loss was measured.[18]

Determination of pH:

In a digital balanced weighing machine, 1 gm of vitamin C powder was weighed and mixed with 20 ml of distilled water. pH of this solution was calculated using a digital pH meter.[19]

Determination of total ash:

2 gm of vitamin C powder was weighed in a digital balanced weighing machine and was taken as a sample into the silica crucible. It was gradually heated with a burner using a 2 cm high flame and supporting the dish about 7 cm above the flame until vapors almost stopped being produced. The dish was lowered, and the heat increased until all the carbon had burned away. After cooling it in a desiccator, it is placed in a balanced weighing machine. Total ash content was calculated and expressed as % w/w of air-dried material.[20]

Acid insoluble ash value:

After assessing the total ash, it was poured into a 250 ml beaker with no ash loss, and 100 ml of diluted hydrochloric acid was added to it. Using a water bath, this solution was boiled for 5 minutes, followed by filtration of the solution and collecting the insoluble matter on an ashless filter paper (Whatman no.41). This filtrate was washed with hot water till getting the neutral filtrate. Transferred the insoluble matter-containing filter paper to the initial crucible and dried it on a hot plate at 600^0c using a muffle furnace for ignition (until it became white ash). Allowed this residue to cool in suitable desiccators for about 30 minutes and weigh without delay. Repeated the process until a constant weight was obtained. Calculated this acid-insoluble ash concerning the air-dried drug. [20]

Determination of water-soluble extractive value:

A digital balanced weighing machine measured 5 gm of air-dried vitamin C powder. These powders were mixed with 100 ml of distilled water in a glass stoppered conical flask. It was set aside for 24 hours with frequent shaking. Rapidly filter it after 24 hrs. A pipette is used to transfer 25 ml of filtrate into a tarred flat bottom evaporating dish, which is then put over boiling water to evaporate it to dryness. Again, evaporated dish dried at 105^0 c in the oven. The weight of this residue was measured after cooling, and the percentage of water-soluble extractive was determined and expressed as % w/w with reference to air dried sample.[20,21]

Determination of alcohol soluble extractive value:

A digital balanced weighing machine measured 5 gm of air-dried vitamin C powder. These powders were mixed with 100 ml of ethanol in a glass stoppered conical flask. It was set aside for 24 hours with frequent shaking. Rapidly filter it after 24 hrs. A pipette is used to transfer 25 ml of filtrate into a tarred flat bottom evaporating dish, which is then put over boiling water to evaporate it to dryness. Again, evaporated dish dried at 105^0 c in the oven. The weight of this residue was measured after cooling, and the percentage of alcohol-soluble extractive was determined and expressed as % w/w with reference to air dried sample.[20,21]

Determination of phytochemical constituents:

Test for the presence of tannin and phenol:

- A test tube was filled with 2 ml of vitamin C powder aqueous extract and a few drops of 5 % FeCl3 solution. Deep blue-black color was observed at the end.
- A test tube was filled with 2 ml of vitamin C powder aqueous extract. It was treated with a few drops of acetic acid solution. The red color persists at the end.

- A test tube was filled with 2 ml of vitamin C powder aqueous extract and a few drops of dilute potassium permanganate solution. Discoloration of the solution was observed at the end. This test was performed for the 1st, 2nd, 3rd and 6th months and was found present each month.[22]

Test for the presence of Flavonoids:

- Shinoda test: We took 2 ml of vitamin C powder aqueous drug extract and mixed it with 5 ml of 95% ethanol. Then it was mixed with a few drops of concentrated HCl and 0.5 gm of magnesium. The pink color was observed.
- Adding an increasing amount of sodium hydroxide to the residue shows yellow coloration, which decolorizes after adding acid. [22]

Test for the presence of carbohydrates:

- Fehling's Test: Take 2 ml of the sample solution in a clean test tube. Add 2 ml of Fehling's solution A and Fehling's solution B. Keep the solution in a boiling water bath for about 10 minutes. If a red precipitate is formed, then the presence of carbohydrates is confirmed.
- Molisch's Test: Take 2 ml of the sample solution in a clean test tube. Add 2-3 drops of Molisch reagent slowly. Now add concentrated sulfuric acid along the sides of the test tube. The acid layer forms a layer at the bottom. Note the junction of the two layers. If there is a formation of the violet ring, then the presence of carbohydrates is confirmed.[22]

Determination of total tannin:

For blank:300 ml distilled water and 25 ml, indigo carmine solution was transferred to a 500 ml conical flask and thoroughly mixed. This solution was titrated against 0.02 M KMnO4 solution until stable golden-yellow color developed and the burette reading was noted.

For sample: Air-dried vitamin C powder was weighed 0.05 gm in the digital balanced weighing machine. This sample was poured into a 500 ml conical flask, and 50 ml of distilled water was added until the sample was fully dissolved. 250 ml sterile water was carefully added to this solution and thoroughly mixed. 25 ml of indigo carmine solution was added to it and mixed well. Carefully titrated this solution against 0.02 M KMnO4 until stable golden-yellow color persisted. The burette reading was noted. The percentage of total tannin was calculated using the following factor. 1 ml of 0.02 M KMnO4 is equivalent to 0.00415 gm of tannin.[23]

4. Microbiological parameters

Determination of total aerobic bacterial count:

Before starting microbial analysis LAF (Laminar Air Flow) and UV (Ultra Violet) lights were switched on for about 30 minutes. Before starting microbial analysis under LAF, UV lights were switched off and daylight was switched on. 10 gm of air-dried vitamin C powder were dissolved and suspended in 90 ml sterile soybean casein digest medium with 4% polysorbate 80 (Tween -80). 1 ml of the solution was pipette out from the above solution using a micropipette and poured into each of the two sterile petri plates. Micropipette tips were discarded in a beaker containing disinfectant in use. Hands were wiped with 70% IPA immediately. 15-20 ml of sterile molten soybean casein digest agar (40-45^{0}c) was poured into the two plates for the total aerobic bacterial count. Plates were swirling slowly to give them uniform dispersion of sample by taking

care that media did not touch the plate lid. Negative control was prepared by pouring 15-20 ml of each used media in a separate empty sterile petri plate and allowing it to solidify at the end of the analysis. Soybean casein digest agar plates were inverted and incubated at 30- 35^0c for 120 hours.[24]

Determination of total aerobic fungal count:

Before starting microbial analysis LAF (Laminar Air Flow) and UV (Ultra Violet) lights were switched on for about 30 minutes. Before starting microbial analysis under LAF, UV lights were switched off, and daylight was switched on. 10 gm of air-dried vitamin C powder were dissolved and suspended in 90 ml sterile soybean casein digest medium with 4% polysorbate 80 (Tween -80). 1 ml of the solution was pipette out from the above solution using a micropipette and poured into each of the two sterile petri plates. Micropipette tips were discarded in a beaker containing disinfectant in use. Hands were wiped with 70% IPA immediately. 15-20 ml of sterile molten sabouraud dextrose agar (40-45^0c) was poured into the two plates for the total aerobic fungal count. Plates were swirling slowly to give them uniform dispersion of sample by taking care that media did not touch the plate lid. Negative control was prepared by pouring 15-20 ml of each used media in a separate empty sterile petri plate and allowing it to solidify at the end of the analysis. Sabouraud dextrose agar plates were inverted and incubated at 20-25^0 c for 120 hours.[24]

Determination of specified microorganisms:

Test for Escherichia coli:

100 ml of Soyabean Casein Digest Broth was prepared and autoclaved for 15-20 minutes at 121^0 c and 15 psi pressure. 10 gm of air-dried vitamin C powder were dissolved and suspended in a sterile 90 ml Soyabean casein digest medium with 4% polysorbate 80 (Tween80). (Solution

A). 100 ml MacConkey's broth was prepared and autoclaved for 15-20 minutes at 121^0 c and 15 psi pressure and then add 1 ml of solution A was added. The above solution was incubated at 43-45^0 c for about 24 hours. Then 100 ml of MacConkey's agar was prepared and sterilized by autoclaving. After sterilization, the agar solution was cooled and poured into the sterile petri dish. Wait until it solidifies. Incubated broth solution was removed and inoculated into solidified agar using an inoculation loop under the laminar airflow. These agar plates were incubated at 37^0 c for about five days. Growth of E- coli was compared with the standard. [24]

Test for pseudomonas aeruginosa:

100 ml of Soyabean Casein Digest Broth was prepared and autoclaved for 15-20 minutes at 121^0 c and 15 psi pressure. 10 gm of air-dried vitamin C powder were dissolved and suspended in a sterile 90 ml Soyabean casein digest medium with 4% polysorbate 80 (Tween80). (Solution A). The above solution was incubated at 43-45^0c for about 24 hours. 100 ml Cetrimide agar was then prepared and sterilized by autoclaving. Wait until it solidifies. Incubated broth solution was removed and inoculated into solidified agar using an inoculation loop under the laminar airflow. These agar plates were incubated at 37^0c for about five days. The growth of Pseudomonas aeruginosa was compared with the standard.[24]

Test for Salmonella species:

100 ml of Soyabean Casein Digest Broth was prepared and autoclaved for 15-20 minutes at 121^0 c and 15 psi pressure. 10 gm of air-dried vitamin C powder were dissolved and suspended in a sterile 90 ml Soyabean casein digest medium with 4% polysorbate 80 (Tween80). (Solution A). The above solution was incubated at 43-45^0c for about 24 hours. 100 ml Rappaport Vassiliadis Salmonella Enrichment Broth was prepared, and 1 ml solution A was added. Again,

the above solution was incubated at 30-35^0 c for 24 hrs. 100 ml of Bismuth sulfate agar was prepared and sterilized by autoclaving. Incubated broth solution was removed and inoculated into solidified agar using an inoculation loop under the laminar airflow. These agar plates were incubated at 37^0 c for about five days. The growth of salmonella species was compared with the standard.[24]

Test for shigella boydii:

100 ml of Soyabean Casein Digest Broth was prepared and autoclaved for 15-20 minutes at 121^0 c and 15 psi pressure. 10 gm of air-dried vitamin C powder were dissolved and suspended in a sterile 90 ml Soyabean casein digest medium with 4% polysorbate 80 (Tween80). (Solution A). The above solution was incubated at 43-45^0 c for about 24 hours. 100 ml of GN broth was prepared, 1 ml of solution A was added, and it was incubated at 30-35^{0}c for 24 hours. 100 ml of Xylose Lysine Deoxycholate agar was then prepared and sterilized by autoclaving. Incubated broth solution was removed and inoculated into solidified agar using an inoculation loop under the laminar airflow. These agar plates were incubated at 37^0 c for about five days. The growth of shigella boydii was compared with the standard.[24]

5. **Estimation of Nutritional Value:** We tested vitamin C powder's nutritional value from the TUV lab in Pune. All **(Table 3)**
6. **Cell viability assay/ MTT assay:**

Human embryonic kidney (HEK293) cells were obtained from the National Centre for Cell Science (Pune). HEK293 cells were cultured in DMEM. The culture media were supplemented with 10% Fetal Bovine Serum, grown in a humidified incubator with 5% CO_2.

Cell viability was assessed by MTT assay. Briefly, HEK293 cells (1×10^4) were seeded into 96-well microplates (flat-bottom), treated with vitamin C powder at 25-1100 μg/ml concentrations, and incubated for 24 h. After 24 h. MTT (0.5 mg/ml) solution was added to each well and incubated for 24 hr. at 37^oC. MTT solution was carefully aspirated and isopropanol was added to dissolve formazan crystals, and the optical density of formazan solutions was recorded at 570 nm using an automated microplate reader (EPOCH2; Bio Tek Instruments, Highland Park, VT, USA). All experiments were done in biological triplicates.

Result and discussion:

Result:

Table 1: Table: Organoleptic Properties of Raw Material

Parameters	Acerola Extract	Rosehip Extract	Moringa Extract	Amla Powder	Bilberry Extract
Colour	Light Red	Light Brown	Brown	Light Brown	Brown
Odour	Characteristics	Characteristics	Characteristics	Characteristics	Characteristics
Taste	Characteristics	Characteristics	Characteristics	Slightly bitter and sour	Characteristics

Table 2: Powder Characterization of Raw Material

Parameters	Acerola Extract	Rosehip Extract	Moringa Extract	Amla Powder	Bilberry Extract
Bulk Density	0.62	0.64	0.63	0.64	0.62
Tapped Density	0.70	0.71	0.70	0.72	0.70
Hausner Ratio	1.12	1.11	1.11	1.12	1.12
Carr Index	11.50	9.90	9.90	11.50	11.50
Angle of repose	40.06	37.23	39.35	40.03	39

Table 3: Solid-State Stability Study Parameters

Sr. No.	Test Parameters	Observations
1.	Caking	Absent
2.	Liquefaction	Absent
3.	Discoloration	Absent
4.	Odor	Absent
5.	Gel formation	Absent

Table 4: Powder characterization of Vitamin C powder formulation

Sr. No.	Test parameter	Result
1.	Color	Light Brown
2.	Odor	Characteristics
3.	Taste	Bitter

Table 5: Powder characterization of Vitamin C powder formulation

Sr. No.	Test parameter	Result
1.	Bulk density(gm/ml)	0.63 ± 0.02
2.	Tapped density(gm/ml)	0.70± 0.02
3.	Hausner Ratio	1.11 ± 0.01
4.	Carr Index	10.86 ± 0.02
5.	Angle of repose	39.39^{0} ± 0.20

Table 6: Physicochemical characteristics of Vitamin C powder formulation

Sr. No.	Test parameter	Result
1	Loss on Drying	8 %
2	Ph	3.87
3	Total Ash	5.73
4	Acid Insoluble Ash	0.43 %
5	Water soluble extractive value	67 %
6	Alcohol soluble extractive value	25 %
7	Determination of Phytochemical constituents	
	a) Tannin	Present
	b) Flavonoid	Present
	c) Phenol	Present
	d) Carbohydrates	Present
8	Essay	
	a) Determination of Total Tannin	21.24 %

Table 7: Microbiological parameters of Vitamin C powder formulation

Sr. No.	Test parameter	Result
1	Total Aerobic Bacterial Count	12 cfu /gm
2	Total Aerobic Fungal Count	7 cfu /gm
3	Specific Pathogens	
	a) Escherichia Coli	Absent
	b) Staphylococcus Aureus	Absent
	c) Salmonella Species	Absent
	d) Pseudomonas Aeruginosa	Absent

Figure 1: Total aerobic bacterial count in SCDA medium

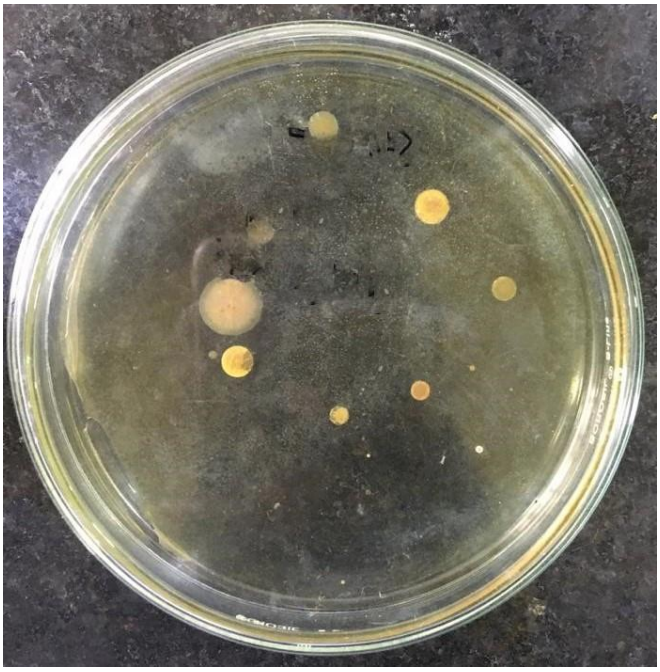

Figure 2: Total aerobic fungal count in SDA medium

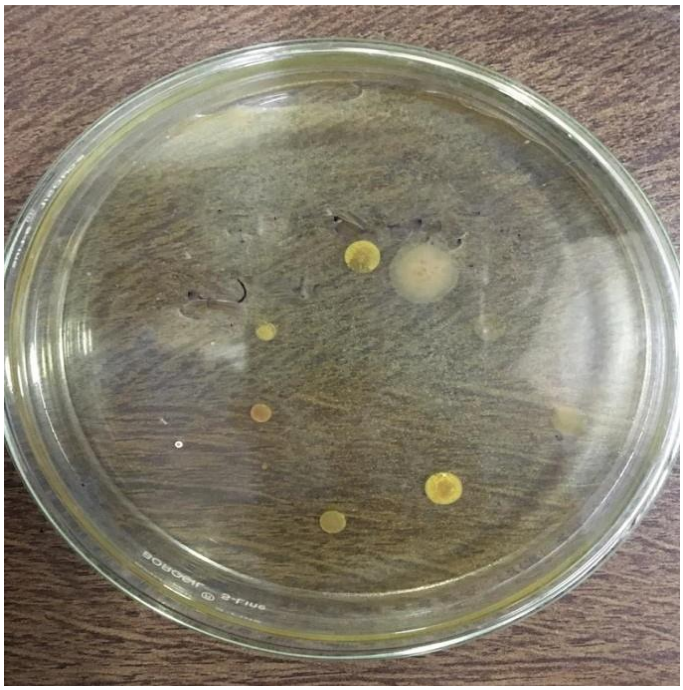

Figure 3: Specific Pathogen test of Escherichia coli in MacConkey agar

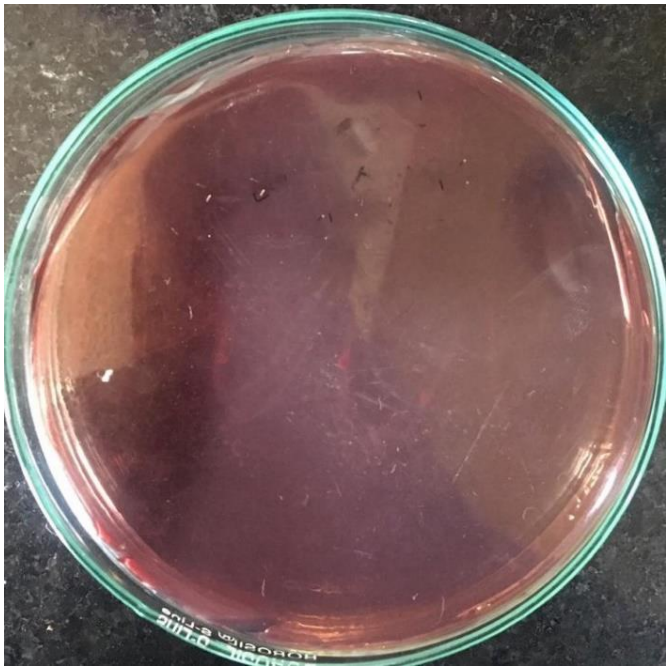

Figure 4: Specific Pathogen test of pseudomonas aeruginosa in cetrimide agar

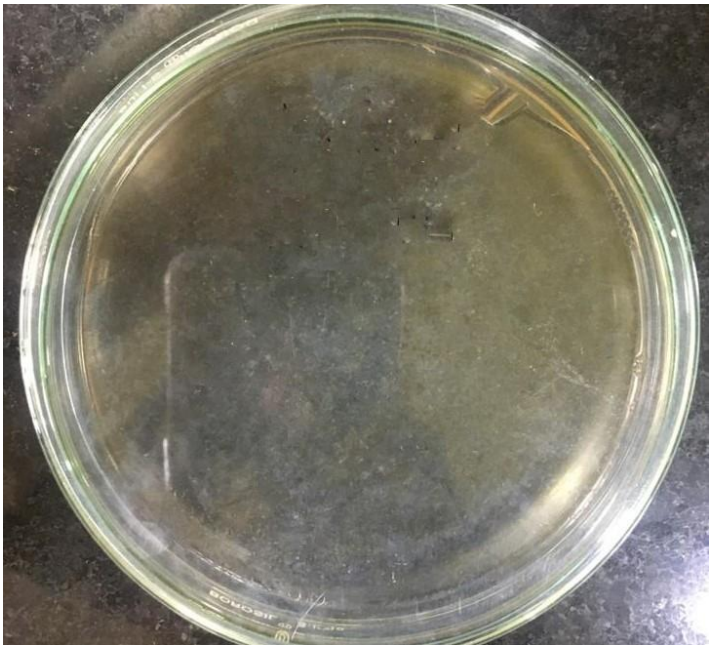

Figure 5: Specific Pathogen test of salmonella species in Bismuth sulphate agar

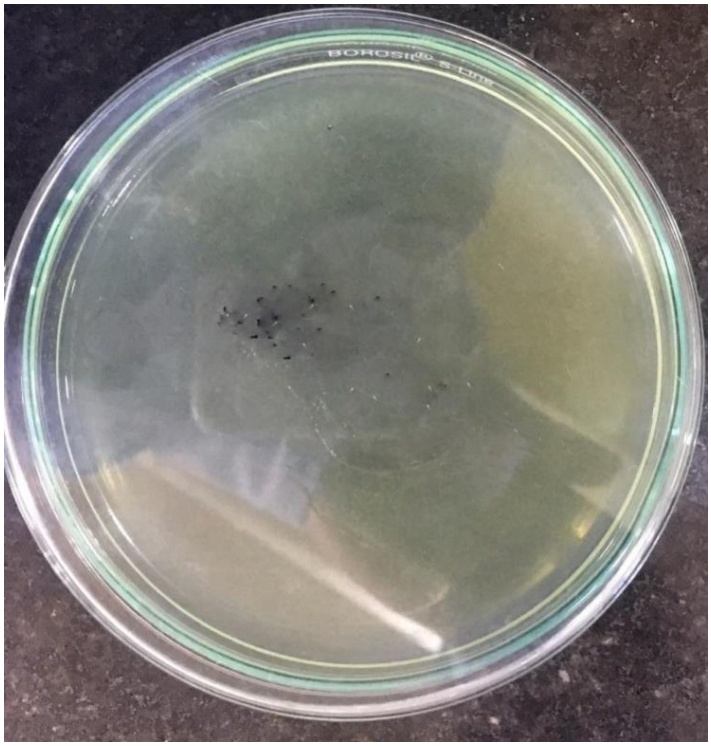

Figure 6: Specific Pathogen test of shigella boydii in Xylose Lysine Deoxycholate agar

Table 8: Estimation of Nutritional Value of Vitamin C powder formulation: -

Nutritional Facts			
NUTRIENTS	**APPROX PER**		**% RDA***
	100 g	**6g**	
Energy (Kcal)	383	22.98	1.03 %#
Total Protein (g)	7.15	0.43	0.94 %
Carbohydrate (g)	79.27	4.76	**
Total Sugar (g)	16.85	1.01	**
Added Sugar (g)	0.00	0.00	**
Dietary Fibre (g)	17.59	1.06	**
Total Fat (g)	0.27	0.02	0.06%#
Cholesterol (mg)	0.00	0.00	**
Vitamin C (mg)	1050.64	63.04	96.98 %*
Potassium (mg)	693.22	41.59	1.19 %*
Sodium (mg)	1270.14	76.21	3.81 %*
Zinc (mg)	0.67	0.04	0.40 %#

* %RDA values established as per ICMR Guidelines-2020.

** %RDA values not established.

%RDA values established as per ICMR Guidelines-2010

Cell viability assay/ MTT assay:

The cell viability of the vitamin C was determined by MTT assay (Fig 7). The IC50 (Inhibitory concentration) value for the MTT assay is 850 mcg/ml, meaning that 50% of cells die at this concentration. It demonstrates that we can give the maximum daily dose of 850 mcg/ml.

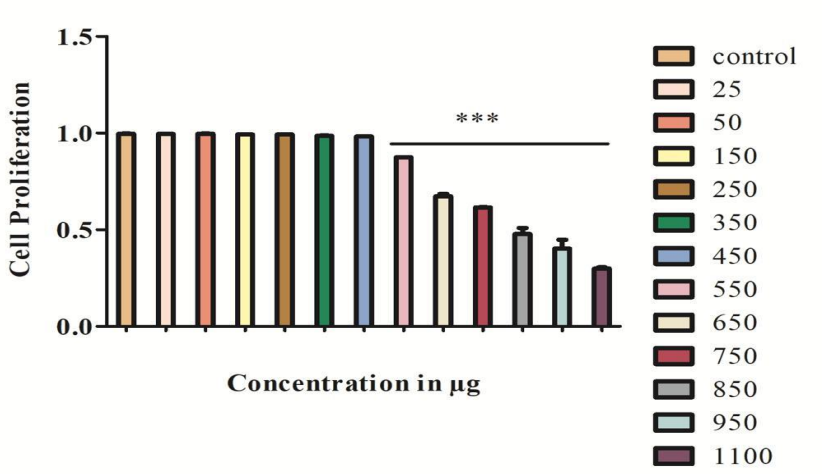

Fig 7. Cell viability for HEK293.

Discussion:

The physical characteristics of the powder were evaluated. The color of the powder was light brown with characteristics of odor and bitter taste. The powder characterization of powder is illustrated in (Table 1). The mean values of bulk density, tapped density, Hausner's ratio, Carr Index, compressibility index, and angle of repose were 0.633 ± 0.02, 0.70± 0.02, 1.11 ± 0.01, 10.86 ± 0.02, 10.25 ± 0.22 and 39.39^{θ} ± 0. 20 respectively. The mean weight loss percentage on drying of vitamin C powder is 8 %. (Table 2) The pH of vitamin C powder was found to be 3.87. A high ash value indicates contamination, substitution, adulteration, or carelessness in preparing the drug or drug combinations for marketing. The total ash value of vitamin C powder was found to be 5.73% w/w. The acid-insoluble ash value of vitamin C powder was 0.43% ± 0.01. The water-soluble and alcohol-soluble extractive values of vitamin C powder were 67% and 25%, respectively. (Table 2) Phytochemical constituents' tannins, flavonoids, phenols, and carbohydrates were present on qualitative estimation tests. The overall tannin percentage was found to be 21.24%. The total aerobic bacterial and fungi count below the permitted range, while specific pathogens were reported to be absent during the study.

The percentage of the recommended daily allowance (%RDA) for nutritional parameters such as energy (Kcal), total protein (g), carbohydrate (g), added sugar (g), dietary fiber (g), total fat (g), cholesterol (mg), potassium (mg), sodium (mg) and zinc (mg) in vitamin C powder formulations is not exceeded, according to ICMR guidelines. Also, it fulfills the daily Vitamin C (Table 3) requirement of adults.

The in vitro cellular cytotoxicity test of vitamin C powder showed no significant cytotoxicity against the HEK293cell line.

Conclusion:

In the present study, a stable vitamin C powder formulation was prepared and evaluated physiochemically and microbiologically. Also, the MTT assay proved that it does not indicate cytotoxicity at the daily dose limit and fulfills adults' daily Vitamin C requirement.

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