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DNA Barcoding, Phytochemical Analysis of *Basella Alba* and its Correlation with Mouth Ulcer

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Abstract: ***Basella alba is one of the popular tropical vegetables grownon the coast of India and most of Southeast Asia. Being a reservoir of nutrients it is not only known for its nutritional value but also for the wide array of medicinal value which has been put into application in traditional medicine systems around the world from olden times. This paper tries to explore these medicinal values with a pharmaceutical vision. Mouth ulcershave been one of the medical conditions that widely impact populations with different agents of causes. We have carried out Epidemiology studies on mouth ulcers using bioinformatics tools. DNA sequencing was carried out to confirm the species. Phytochemical analysis of B. albaleaf extract was carried out to confirm the presence of phytochemicals like flavonoids, polyphenols, and terpenoids which are known to be effective in the treatment of mouth ulcers to establish the scientific reason behind using B. alba in the mouth ulcer treatments in ethnomedicines and tested polysaccharide contents with a perspective to inculcate B. alba in the ointment formulations for mouth ulcer.***

Keywords: Antiulcer property, ethnomedicines, flavonoids, polyphenols, Sanger sequencing.

1. Introduction

Basella alba belongs to the family of the edible perennial vine, Basellaceae. It is a leafy vegetable found in the tropical regions of Asia and Africa. It originated from the Indian subcontinent, Southeast Asia and New Guinea. It was later grown in China, tropical Africa, Brazil, Belize, Colombia, the Philippines, the West Indies, Fiji, and French Polynesia. [1]. Malabar spinach, vine spinach, and Ceylon spinach are the common names of *Basella alba*. It is widely cultivated as a cool-season vegetable with a climbing growth habitat. It is a succulent, smooth, twining vine, several meters in length. Stems are either purplish or color. Leaves are heart-shaped, fleshy, and grow up to 5-12cm long, tapers at the end as a pointed tip. Fruits are ovoid in shape and grow up to 5-6mm long and have characteristic purple color once they fully mature [2]. The leaves and stems have been significantly used for their medical properties of which one known study is about the use of leaf extract to treat mouth ulcers in traditional medicine systems around the world. In this paper, we try to explore the scientific reasoning behind utilizing the *Basella alba* leaf extract at first we carried have carried out DNA sequencing to confirm the sample that we have taken is *Basella alba* followed by studying the quantitative compositions of the phytochemicals such as flavonoids, polyphenols which are already known to have antiulcer activity as well as studying the antibacterial property against *Pseudomonas aeruginosa* and *Streptococcus aureus* which are known to cause mouth ulcer. We have also studied the polysaccharide composition in order to explore the application of the leaf mucilage as a binder in ointments for mouth ulcer treatment ulcer.

Figure 1: Leaves (A) Flowers (B) and fruits (C) of *B. Alba*

2. Literature Survey

Basella alba has a wide range of medical applications. Ithas been used as a treatment for anemia in women. The stems and leaves have been used to treat colds and coughs [3]. Leaves have been used for constipation, urticaria, and gonorrhea [4]. In the Indian traditional medicine system, it has been used to treat burns [5] as well as acne and freckle treatments in Bangladesh. In Nepal, leaf juice has been used to treat Dysentery. The mucilage of leaves and stalks have been used as a remedy for headache [6]. Maceration has been administered orally in cases of infertility, risk of abortion, and Braxton Hicks contractions during the last days of pregnancy [7]. *Basella alba* is known to be a rich reservoir of nutrients. It contains high amounts of Vitamin A, Vitamin C, and iron. A population study conducted on

Bangladeshi men showed that regular consumption of *Basella alba* had a positive impact on vitamin A levels in the blood [8]. Its antidiabetic property was validated in mice models by studies conducted by [9] wherein it was found that *Basella alba* extract increased levels of Glutathione-S-transferase (GSH) a non-enzymatic tissue anti-oxidant. GSH reduced oxidative stress which in turn helped in alleviating diabetes. The anticholesterolemic property of *B. Alba* was validated in the rabbit model studies conducted by Baskaran et al., 2015 [10]**.** In this study, *B. alba* leaf extract was evaluated in comparison to Simvastatin and found to have anticholesterolemic activity, without the adverse effects found in Simvastatin. The antimicrobial activity of *Basella alba* was evaluated against *Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli* in the studies conducted by Oyewole et al., 2012 [11]. The anticancer property of leaves and seeds of *B. alba* was confirmed against the Ehrlich's ascites carcinoma (EAC) cell line in the studies conducted by Islam *et al*., 2018 [12]**.** In studies conducted by George *et al.*, 2018 the anthelminthic property of *Basella alba* was confirmed against *E. Fetida*, wherein Albendazole was used as standard [13]. The mucilage of the *Basella alba* flower has been used as an artificial saliva formulation [14].

A mouth ulcer (aphtha) is a form of ulcer that forms on the mucous membrane of the oral cavity. Mouth ulcers mostly occur in association with many diseases and by many different mechanisms, but usually, there is no serious underlying cause. In rare cases, mouth ulcers that do not heal in time can be indications of oral cancer. Ulceration is a common clinical manifestation that is caused due to many factors. These factors can be categorized as infections, nutritional deficiency, specific diseases, immunology, and genes. This involvement of many factors makes the diagnosis of mouth ulcers challenging.

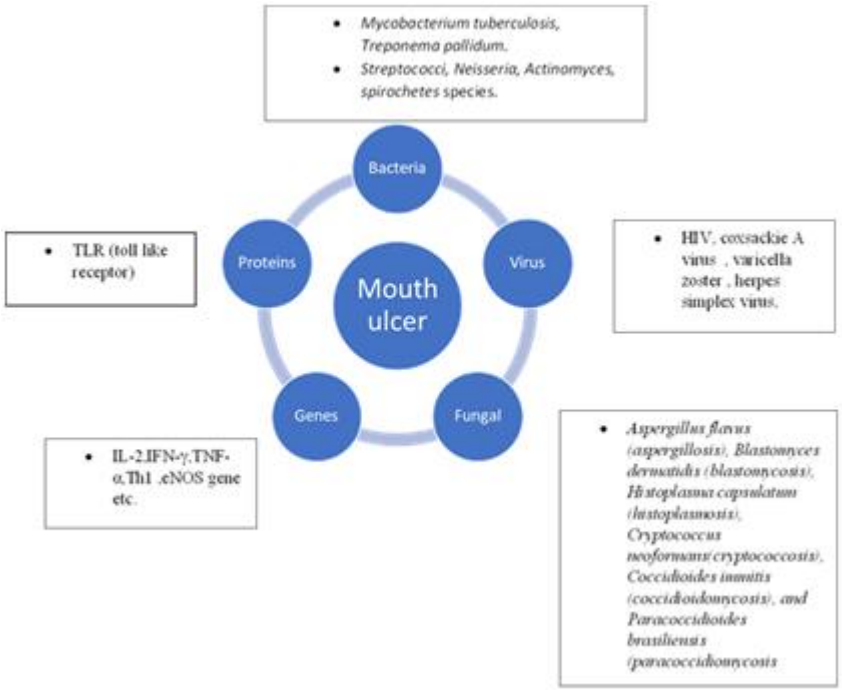

Figure 2: Etiology of mouth ulcer.

Bacterial infection by *Mycobacterium tuberculosis* (tuberculosis) [15] and *Treponema pallidum* are known to cause mouth ulcers. Normal bacterial flora, such as aerobic *streptococci, Actinomyces, Neisseria, spirochetes*, and Bacteroides species under opportunistic conditions can lead to mouth ulcers. In the mouth ulcers causing virus the most common ones are the herpes simplex virus which is responsible for *herpes labialis*, primary *herpetic gingivostomatitis, coxsackie*, a virus that causes hand, foot, and mouth disease, *Varicella zoster* the virus that leads to chickenpox and shingles. Various fungal infections causing oral ulcerations include *Aspergillus fumigatus* or *Aspergillus flavus* (aspergillosis), *Histoplasma capsulatum* (histoplasmosis), *Blastomycesdermatidis* (blastomycosis), *Coccidioidesimmitis* (coccidioidomycosis), *Cryptococcus neoformans (cryptococcosis*), and *Paracoccidioidesbrasiliensis* (paracoccidioidomycosis). These infections mostly occurred in individuals with compromised immunity. In the case of aspergillosis ulcerations, the lesions appear as black or yellow and commonly affected areas are the tongue or palate. *Blastomycosis* might cause oral ulcers which can appear to be similar to squamous cell carcinoma. The HIV virus is not directly involved with causing the mouth ulcer however it has a damaging effect on the immune system, which in some cases leads to mouth ulcers.

The elevated concentrations of mRNA corresponding with IFN-γ, IL-2, and TNF-α and the decreased mRNA level with respect to the IL-10 were detected in subjects with aphthae in comparison to healthy controls (Akeman *et al*., 2006, observed a higher frequency of TNF-α1031C allele, analogous with the increased number of mononuclear cells that produce TNF-α and IFN-γ in peripheral blood of patients with Bechet's syndrome in comparison to healthy controls [17]. Studies by Borra *et al*., 2004 showed that in RAS patients, that increased expression of the TH1 gene in comparison to the Th2 is the key immune response related to the progression of the disease [18]. This type of increased expression of Th1 type immune response was also found in

autoimmune diseases such as celiac disease, Crohn's disease, and PFAPA. Some studies suggest the role of endothelial nitric oxide synthase (eNOS) gene polymorphism in the development of RAS. Many biological reactions are mediated by Nitric oxide. It is involved in the conversion of GTP to cGMP, a compound involved in vasodilation, muscle relaxation, and inhibition of platelets and monocyteadhesion. In patients with recurrent aphthous stomatitis, a higher incidence of HLA-B35, HLA-A33 and HLAB12, HLA-B81, HLA-DR5, and HLA DR7 and lower incidence of HLA-DR4 and HLA-B5 was observed when compared to healthy controls [19]. However, they were unsuccessful in demon strating the correlation between the individual HLA alleles and RAS in the examined subjects, although it was proved that in concordance with the HLA haplotypes Ras susceptibility was inherited.

3. Materials and Methods

Sample collection
The leaf samples were collected from the local house garden in Bangalore, India. The leaves were isolated from the stocks and cleaned with tap water and air-dried.

DNA isolation
The isolation of DNA was carried out using the CTAB method.100mg of B. *alba* leaves were added to 500µl of CTAB extraction buffer followed by through vortex and then transferred to a hot water bath at 60ºC for 30mins. After the incubation period, the mixture was centrifuged at 1400rpm for 5 minutes. An equal volume of chloroform: isoamyl alcohol was added to the sample and then vortexed for 5 seconds followed by centrifugation for 5mins at 14000rpm. The aqueous upper layer was transferred to a new tube. The DNA was precipitated by adding 0.7ml cold isopropanol and incubated at-20ºC for 15mins. Next, the content was transferred to a silica-based DNA column (750µl each time) and spun at 10000rpm for 1min after which a wash buffer of 700ml was added and spun at 10000rpm for 1min. The washing step was repeated now with 600µl of the wash buffer. This was followed by subjecting the content to dry spin for 3mins at 10000rpm. The 20µl of elution buffer was added to the mixture left for 5mins and then subjected to spinning at 10000rpm for 1 min.1µl of RNase was added and incubated at 37ºC for 30mins. The DNA obtained from this procedure was further utilized for quantification.

DNA quantification
DNA quantification was carried out using UV Spectrometric analysis.1ml of TE buffer was taken in a cuvette is calibrated at 260nm as well as 280nm. The isolated DNA of *B. alba* of 10µl was added to Tris EDTA buffer and mixed well. The OD was taken at 260nm and at 280 nm. The ratio of OD at 260 and 280 was calculated. The amount of DNA was quantified using the formula-DNA concentration= ((OD260 *100 (dilution factor) *50µg/ml) /1000)

DNA barcoding
The DNA sample was subjected to gel electrophoresis to confirm the presence of DNA, followed by PCR amplification using ITS2 as forward and reverse primers. The amplified DNA was subjected to gel electrophoresis. The DNA bands were cut from the gel and subjected to gel elution followed by the Sanger sequencing using an automated Sanger's sequencer. The FASTA sequence obtained from the Sanger sequencing was subjected to multiple sequence analyses using the BLAST and phylogenetic analysis was carried out using clustal omega to identify the specimen and its closely related species.

Phytochemical analysis

Ethanol Extract preparation
For the ethanol extract preparation, 10gms of the leaf sample was added with 50ml of ethanol left undisturbed at room temperature for 2 days after which the liquid was separated from the solids and used for further phytochemical analysis.

Estimation of Total phenolic acid content
Gallic acid standards were prepared for concentrations of 20, 40, 60, and 80µl as stated in Aryla *et al*., 2019. For the sample preparation, 250µl of plant extract was added to 750µl of FC reagent. The final volume was made up to 5ml with distilled water and allowed to stand for 90 minutes at room temperature. The absorbance for samples and standards was noted at 760 nm. [20]

Total flavonoid estimation
Quercetin standards were prepared for varying concentrations of 0.25, 0.5, 0.75, 1 mg/ml. For the sample, 1 ml of the ethanolic leaf extract was taken. At the same time, 0.3 mL of 5% $NaNO_2$ was added to the test tube and 0.3 mL of 10% AlCl3 after 5 min. This was followed up by the addition of 2ml of 1M NaOH to the mixture after 6 minutes and the volume was made up to 10ml. The absorbance for the standards and the test sample was noted down at 510 nm [21-22].

Polysaccharide content estimation
The glucose standard curve was prepared for 0, 80, 160, 240, 320, 400 µg/ml concentrations of glucose.1ml of 5% phenol was added to the 1ml of ethanol extract of *Basella alba*leaf followed by 5ml of concentrated H_2SO_4. The absorbance was measured after 10mins at 488nm against blank for the standards as well as the sample.

Qualitative assessment of terpenoids.
1ml of the ethanolic leaf extract sample was added with 2-3 drops of acetic acid and stirred slowly until it dries. This was followed by the addition of 1-2 drops of conc H_2SO_4. The color of the mixture was observed.

Antibacterial test.
Pseudomonas aeruginosa and *Streptococcus aureus* were chosen for the antibacterial test as they are one of the causative bacterial species for mouth ulcers. The bacteria were inoculated onto nutrient agar. The ampicillin was used as a positive control and the ethanol was used as negative control along with the *B. alba* ethanol extract as the test sample. The incubation was carried out at 32ºC for 24hrs. The size of the zone of inhibition was noted down.

Statistical analysis

The trials were carried out in triplicates for quantification experiments. The values obtained from the experiments were analyzed for average and the SD error using Microsoft excel.

Bioinformatic research on mouth ulcers and establishing a correlation with causes of mouth ulcers to the scope of *B. alba* as a treatment of mouth ulcers.

The literature study was carried out on mouth ulcer causes using NCBI and the phytochemicals that are used for curing mouth ulcer was searched.

4. Results

Quantification of DNA

OD at 260nm was found to be 0.6 and OD at 280nm was found to be 0.034. The ratio of OD 260 nm and 280nm was found to be 1.8 which confirmed that the sample was pure.

DNA barcoding

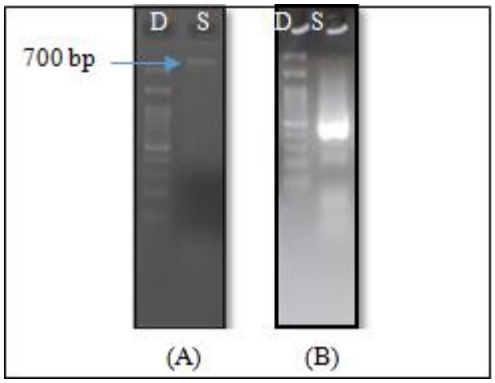

Figure 3: Results of gel electrophoresis of DNA quantification (A) and PCR amplification (B). * D-DNA ladder S-Sample DNA

From the gel electrophoresis result, the size of the DNA was found to be 700bp. The FASTA sequence obtained was as given below –

>BA_ITS2
ATTGTCGAAACCTAGTGCCCAGCAGAATGACCCGC
GGACGAGTTTCACGCACAAGACGCGCGGGGAGGTC
GCCTCCCCCGCGGCCGCACGGCGCCAACCCCCGGT
GTGGCACGTTGCGGCAACAAACCCCGGCGCGGACC
GCGCCAAGGAACACGAAGAGCGAGAGCGCCGGCC
CGTGCCCGTGCCCGGTGCGATGGGCGGCGCCCCAC
ACTAGAAAACGTAACGACTCTCGGCAACGGATATC
TCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG
CAGCTTAGCGATACTTGGTGTGAATTGCAGAATCCC
GTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC
GAAGCCTTCCGGCCGAGGGCACGTCTGCCTGGGCG
TCACGCATCGCGTCTCCCTCACCCGCCGCGCGGGG
GGGAAGGACGATGGCCTCCCGTGCTTGAACGGGCG
CGGCTGGCCTAAAACGGGAGCTTGCGGCGACAGCT
GCGGCGGCGTTTGGTTGACGAGCTGTCGGCCCTCGT
AATGCATCGCGCCTCGCACGCACGTCGTCGGCATG
GGCTGG

Hit obtained from BLAST of the obtained sequence was *Basella alba* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA had a percent identity of 96.86% with E value 0.0 and query coverage of 99%. From the phylogenetic tree analysis, it was found that the test sample was confirmed to be *Basella alba* and is also closely related to *Anrederacordifolia* and *Basella excavate*.

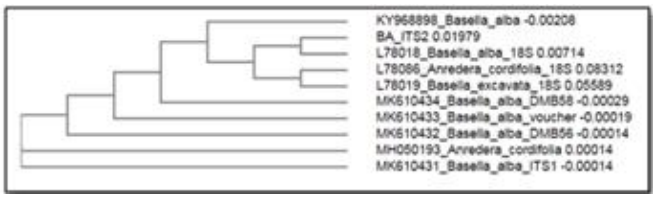

Figure 4: Phylogenetic tree construction.

Ethanol extract preparation.

The ethanol extract was prepared as stated in the above methodology by filtering through the Whatman filter paper so that the solid leaf residues are separated from the extract.

Figure 5: Ethanol extract preparation from *Basella alba* leaf.

Total phenolic content estimation

Gallic acid standard plot

The gallic acid standard curve was plotted as given below for various concentrations of gallic acid and the concentration of the gallic acid in *Basella alba* was found to be 44.99 ±0.251µg/ml. The absorbance was noted down at 760 nm for this experiment for both the standards and test samples.

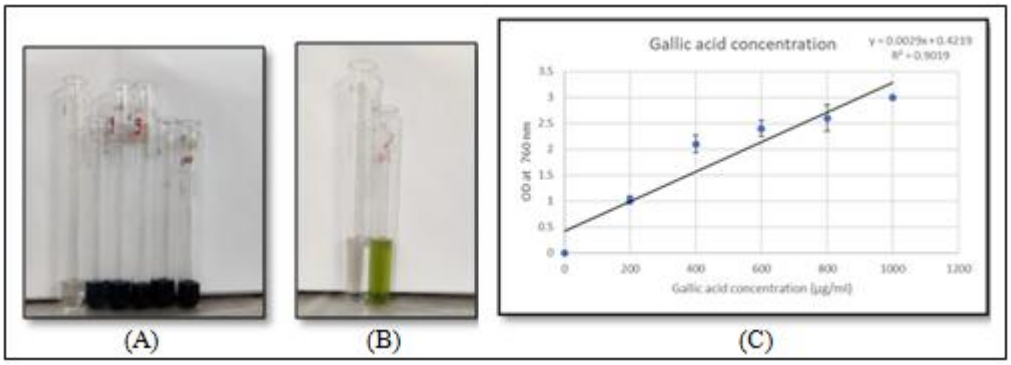

Figure 6: A) Gallic acid standard B) Blank vs *Basella alba* extract C) Gallic acid standard plot.

Quercetin estimation

The Quercetin standard curve was plotted for various concentrations of quercetin as given below and the concentration of the gallic acid in *Basella alba* was found to be 1169.4 ± 0.173µg/ml.

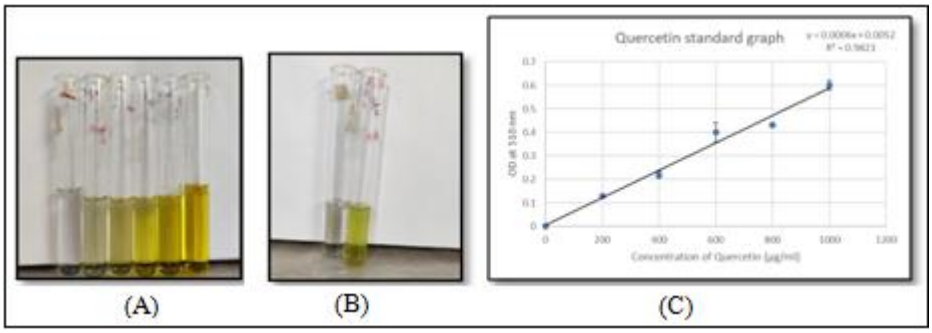

Figure 7: A) quercetin standard B) Blank vs *Basella alba* ethanol extractC) Quercetin standard plot.

Polysaccharide estimation

The standard curve was plotted for glucose concentrations of 80, 160, 240, 320 and 400 µg/ml. The graph was plotted as given below. The polysaccharide content in B. alba extract was found to be 473.73±0.112µg/ml. The absorbance for the standards and the test samples were noted at 490nm.

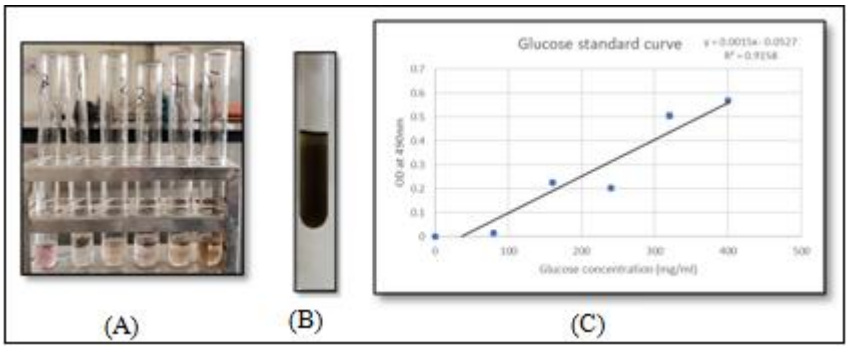

Figure 8: A) Glucose standards B) *B. alba* sample C) Standard glucose curve

Qualitative test of steroid and terpenoids

The reddish-brown interface formed at the end of the reaction indicates a positive result for the presence of terpenoids.

Figure 9: Results of qualitative analysis of terpenoid content in *B. alba* extract.

Antibacterial test

The antibacterial test was carried out by inoculating the solidified sterile agar plates one with *Pseudomonas aeruginosa* and one with *Staphylococcus aureus*. The individual well was loaded with ampicillin being a positive control, 70% ethanol being the negative control, and test samples as 100% extract and 1: 1 dilution of the extract.

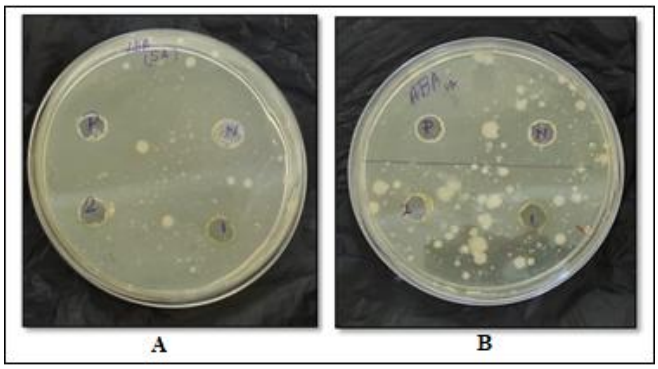

Figure 10: Results for Antibacterial test A) *S. aureus* B) *P. aeruginosa*

Figure 11: Table of length zone of inhibition (PA-*Pseudomonas aeruginosa*, SA-*Staphylococcusaureus*), P-Ampicillin-Ethanol 70%)

Organism	P (cm)	N (cm)	1 (Extract)	2 (1: 1 dilution)
PA	3±0.5	-	1±0.8	1.5±0.2
SA	3±0.2	-	2±0.6	1.5±0.3

Bioinformatic study of causes of mouth ulcer its remedy and correlation with *Basella alba*

From the bioinformatics, studies attempt has been made to establish a correlation between the phytochemicals present in the *Basella alba* and the treatment of mouth ulcer Flavonoids such as quercetin has antiulcer properties. This antiulcer property is contributed due to the antioxidative property of the flavonoids. Quercetin was found to suppress TNF-α and IFN-γ which are the genes responsible for mouth ulcers. Gallic acid has been found to have antioxidant and antimicrobial properties which contribute to treating mouth ulcers caused by microbial infections and ulcers related to oral cancer. Gallic acid scavenges reactive oxygen species and induces apoptosis of cancerous cells hence blocking the growth of ulcers caused by cancerous cells. Terpenoids have a cytotoxic effect on cancerous cells which contributes towards their antiulcer property and have been evaluated in mice model studies. Vitamin B2, Vitamin B12, Vitamin C, zinc, iron, and folic acid deficiency have been found to cause mouth ulcers. *B. alba* being rich in all these nutritional components can cure mouth ulcers caused due to nutritional deficiencies.

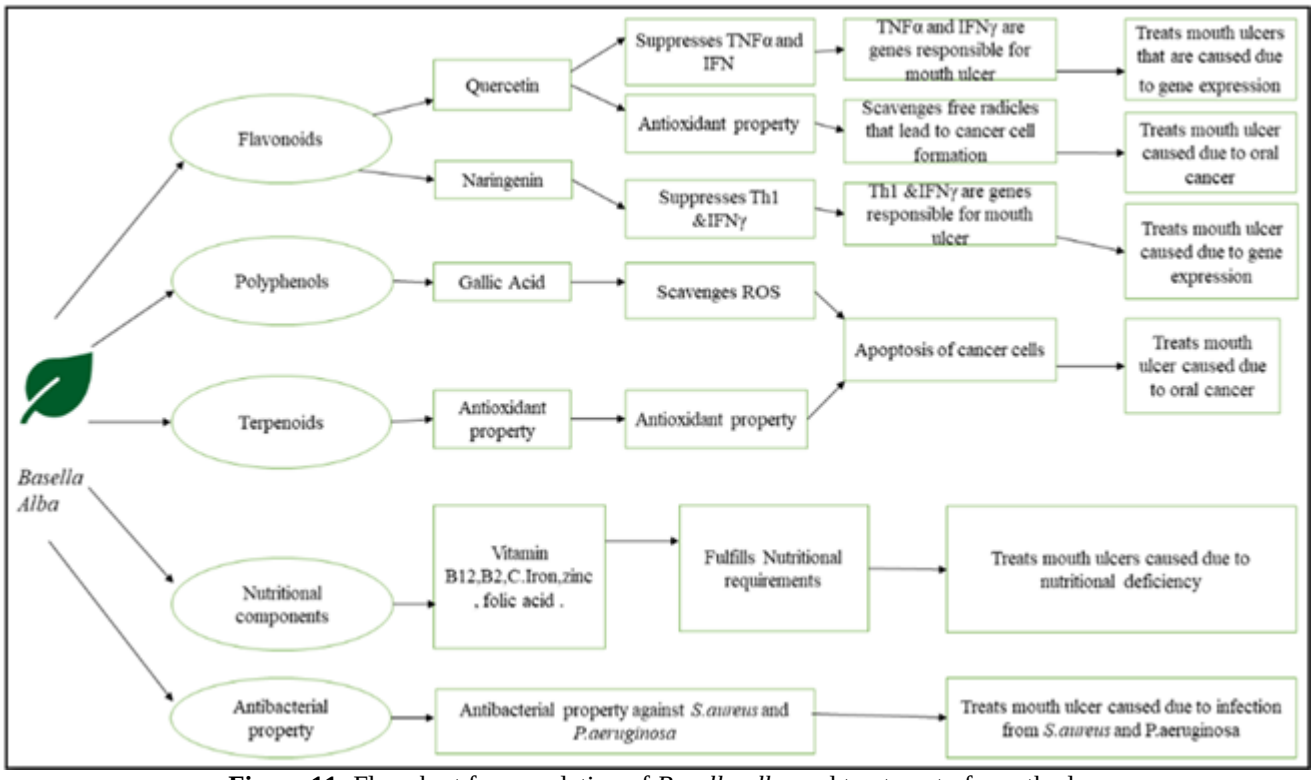

Figure 11: Flowchart for correlation of *Basella alba* and treatment of mouth ulcer.

5. Discussion

From the DNA barcoding, it was confirmed that our sample was *Basella alba*. The phytochemical analysis conducted showed the presence of flavonoids, phenolic content, polysaccharides, and terpenoids. The total phenolic content in the *B. alba* sample in our studies was found to be 44.99µg/ml (0.225 mg QUE/g of dry biomass), while in studies conducted by [22] it was found to be 93.89 mg QUE / g of dry biomass which is comparatively high. The flavonoid content was found to be 1169.4µg/ml (5.874mg GAE/g of dry weight) from our experiment. However, in studies conducted by [23] total phenolic content was found to be 0.74g GAE /kg of dry weight. The polysaccharide content was found to be 811.44µg/ml (4.057mg/g of dry weight) these polysaccharides have a wide range of applications as tablet binders and ointment formulation [24]. The presence of terpenoids was confirmed by a qualitative test. Terpenoids are known to have antimicrobial, antifungal, anti-inflammatory, and immunomodulatory properties [25]. *P. arigenosa* and *S. aureus* are among the bacterial species that are related to causing mouth ulcers [26]. The antibacterial test carried out proved there is little antibacterial property of *B. alba* against *P. arigenosa* and *S. aureus* which can be increased by increasing the concentration of the extract. From the bioinformatics

studies, it was found that there are many factors contributing to the mouth ulcer such as infections by viruses, bacteria, and fungal species of different strains, genes, and proteins linked to mouth ulcers which have been mentioned before. The flavonoids and phenolic contents are found to be effective in treating mouth ulcers caused due to oral cancer as they have antioxidant properties which scavenge free radicles and hence inhibit the growth of cancerous cells.

6. Conclusion

The main purpose of this work is to highlight the medical significance of *Basella alba* which has been widely used in traditional medicine systems and how it can be brought into the mainstream pharmaceutical application with a focus on using it as a treatment for mouth ulcers. From the phytochemical analysis, we were able to estimate polyphenols and flavonoids that have anticancerous properties which would be favorable to cure mouth ulcers caused due to cancer. The plant extract also exhibited the presence of terpenoids which has anti-inflammatory and antiallergic properties and can treat mouth ulcer caused by allergic responses. The presence of polysaccharides opens up the scope of application of *B. alba* mucilage as a tablet binder or as a thickener in ointments.

7. Future Scope

This study can be further elaborated with mouse model studies to confirm if *B. alba* could be an effective treatment for mouth ulcers. Using proteomics novel proteins that are present in *B. alba* can be identified, docked with proteins related to cancer or virus, and can be evaluated for their potential in anticancer and antiviral drug formulations. Genetic engineering could be implemented to increase the phytochemical contents which are of high pharmacological values.

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