How to Cite:
Keerthana, N. A., Basalingappa, K. M., Gopenath, T. S., Parthiban, R., Rajashekara, P. S., Ashok, G., Karthikeyan, M., Mehenderkar, R., Bakthavatchalam, P., Rajasurian, D., Ashok, I., Stephenie, S., & Devi, S. (2022). Edible vaccines: A novel approach to oral immunization and their application in clinical trails. *International Journal of Health Sciences*, *6*(S3), 9402–9421. https://doi.org/10.53730/ijhs.v6nS3.8248

Edible vaccines: A novel approach to oral immunization and their application in clinical trails

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International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2022.
Manuscript submitted: 27 March 2022, Manuscript revised: 9 April 2022, Accepted for publication: 18 May 2022

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***Abstract*---**Infectious diseases cause more than one million deaths every year. Fifty percent of these diseases are caused by bacteria that infect the mucosal membrane of the mammalian host. Vaccines are recognized worldwide as one of the most effective resources against infectious diseases. It is a biological product that can improve the immune response to specific diseases. Edible vaccines are referred to the use of edible parts of the genetically modified plants. It effects on the lining of the gastrointestinal tract allows the activation of systemic immunity and mucosal immunity (GIT). Edible vaccines are used to prevent various diseases, such as hepatitis B, measles, malaria, cholera, Norwalk disease, anthrax, foot-and-mouth disease, rabies, rotavirus, HIV, HPV, diabetes, and sexually transmitted diseases. The purpose of this review is to introduce edible vaccines as a novel oral immunization method, the types and uses of edible vaccines in clinical trials.

***Keywords*---**edible vaccines, transformation, transgenic plants, immune response, clinical trials, oral immunization, recombinant.

Introduction

The main cause of morbidity and mortality in human lives is infectious diseases. More than a million individuals die each year due to various communicable diseases. Around half of these infections are caused by pathogens that damage the host mucosa(1). The most important challenge in the field of medicine is to find a particular vaccine that can target and kill the pathogen at various stages of infection. Vaccines are one of the ground-breaking discoveries in the history of medical sciences to combat infectious diseases. A vaccine is a biological preparation which enhances the immune responses to a specific disease or pathogen (1). Morethan 200 years lapsed since the discovery of first vaccine. In 1796, the vaccination concept was first proposed by Edward Jenner, for smallpox{Gunasekaran, 2020 #56}. The mechanism by which the body is prepared to combat infections is vaccination. This method of treatment is in sharp

contrast to the conventional method of treatment that typically takes place after the onset of a particular disease. Vaccines not only prepare us against any potential infection, but also immunize us for a very long time against those diseases. The evolution of vaccines has contributed to the discovery of new, efficient ways of vaccination covering a broader variety of diseases.

- Live-attenuated vaccines: the original and the 1st vaccines are considered. As a vaccine, the weakened form of a live infectious organism is used.
- Inactivated vaccines: The debris of a dead organism is used as a vaccine in this type of vaccine.
- Toxoid vaccines: This is where the toxin created by the body is used as a vaccine. Toxoid vaccines focus on the prevention, rather than the infection itself, of the ill effects of the infection.
- Biosynthetic vaccines: as the name implies, they are man-made vaccines and have infectious organism-like shapes and properties.
- DNA vaccines: These are plasmid DNA with antigen-encoding sequences. This plasmid DNA is then directly added to a particular muscle or tissue where it is expressed.
- Recombinant vaccines: These vaccines contain a recombinant plasmid carrying the antigen-encoding gene expressed in bacteria. This protein is then purified as a vaccine and used.
- Edible vaccines: the edible portion of a plant is genetically engineered to express antigens, giving rise to an immune response when ingested. [2]

Over-view of edible vaccines

Genetically modified plants and animal-derived medicines containing active ingredients that can trigger an immune response in animals are nothing but edible vaccines. Edible vaccines are foods that contain nutrients. As the name suggests, these nutrients not only provide nutrition, but also function as vaccines to make users immune to different diseases. They are produced to be administered orally to activate the immune system [4]. In 1989, Hiatt and colleagues began to develop plant-dependent vaccines [7]. In 1990, Ph.D. Arntzen first proposed the use of genetically modified plants to produce and distribute subunit vaccines. The expression of hepatitis B surface antigen in tobacco plants has become an important milestone in the development and production of edible vaccines [8]. While producing edible tobacco vaccines, hepatitis B and heat-labile toxin B have also been produced on potato plants [7]. The edible vaccine is made by injecting selected genes into plants. The resulting modified plants induce the production of encoded proteins, which is called the transformation stage, and the resulting plants are called transgenic plants. Various transformation strategies for vaccine vectors derived from plants, bacteria and algae are described in Table1. These vaccines lack pathogenic genes, but they produce antigenic proteins. Therefore, they can guarantee protection, especially for people with weakened immune systems. Edible vaccines can improve compliance, especially in children. It can be easily expanded through an efficient manufacturing process. For example, an area of 40 hectares is enough to produce hepatitis B antigen required for the whole of China to be vaccinated every year [17].

Table 1
Transformation methods in plants, bacteria and microalgae [2].

Transformation method	Plant	Bacteria	Microalgae
Agrobacterium mediated gene transfer	✓		✓
Electroporation	✓	✓	✓
Biolistic/ Gene gun method	✓		✓
Heat-shock		✓	
Electrospray			
Glass beads			✓

Characteristics of an optimal vaccine

- It should be non-pathogenic and therefore stable.
- It should cause the least side effects on healthy individuals.
- It should elicit the production of long-lasting cellular and humoral immune responses.
- It should have good genetic stability.
- It should not pose problems in immuno-compromised patients.
- It should have less ease of contamination.
- It should be effective and affordable [9-11].

In 1998, edible vaccines–were approved by the National Institute of Allergy and Infectious Diseases for their unique immunogenicity effect. These vaccines offer a cost-effective, simple, needleless, safe, convenient and better alternative to vaccine manufacturing[14,15,18]. The following are the advantages offered by Edible Vaccines over the conventional forms of vaccines.

Advantages of Edible Vaccines

- Economical in mass-cultivation.
- The steps of processing and purification can be eliminated as they are administered via the oral route.
- Conditions of cold storage are not required as they are heat stable and can be stored at normal room temperature.
- Capital-intensive pharmaceutical manufacturing facilities are not required for their production.
- They activate the first line of defense.
- Pathogen and toxin-free therapeutic proteins.
- Medical staff standards and sterile conditions are minimized.
- Bio-encapsulation ensures antigen protection.
- Multiple antigens can be delivered and they undergo seroprotection in the presence of specific antibodies.

Disadvantages of Edible Vaccines

- Difficulty in selecting plants with sufficient antigen production.
- The task of dosage evaluation is tedious.

- Differences in dosage consistency from plant-to-plant as well as from generation-to-generation.
- Possibility for the development of oral tolerance as well as hypersensitivity reactions towards the vaccines.
- The cooking of food containing vaccines may weaken its medicinal properties.
- Vaccine stability in fruits is unknown [6, 17, and 18].

Mode of action

Edible vaccines allow both systemic and mucosal immunity to activate upon contact with the GIT lining. This dual effect is provided by the first line of protection against pathogens that invade through the mucosa [17]. Bio-encapsulation (the outer, tough plant cell wall) delivers the antigens released in transgenic plants, ensuring antigen defense from gastric secretions. They are then eventually split up in the gutThe released antigens are taken up by M-cells in the intestinal lining overlying Payer's patches and gut-associated lymphoid tissue (GALT) and transferred to antigen-presenting cells such as macrophages and local lymphocytes. This contributes to local IgA response production, serum IgG, IgE responses, and memory cells, all of which neutralize the attack of the true causative agent [12].

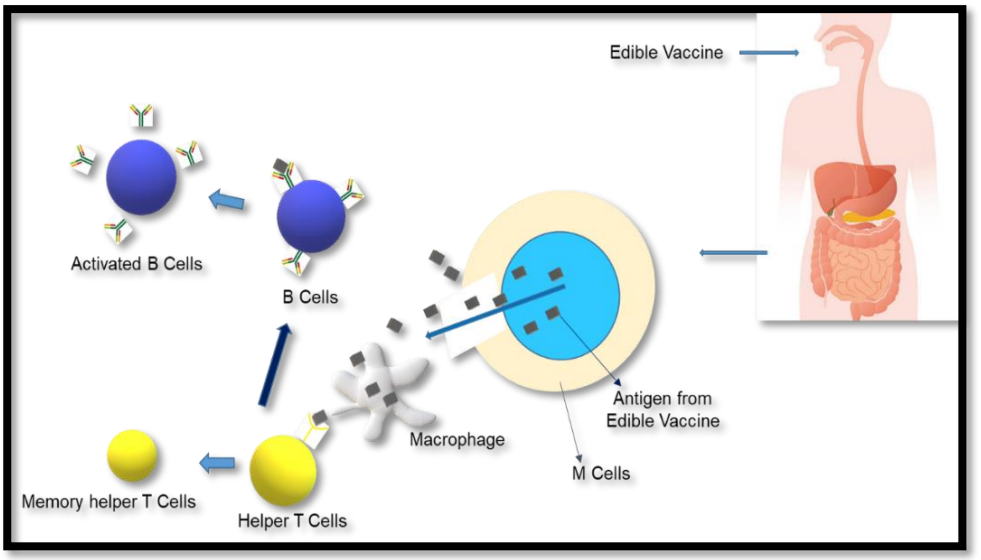

Figure 1. Mechanism of effect of edible vaccines

Types of edible vaccines

Edible vaccines are divided into the following categories, depending on the source of the expression system used to produce a vaccine:

Plant-based edible vaccines

In recent years, plants have been widely used for the creation of novel biopharmaceutical production platforms because they facilitate proper folding of

exogenous proteins and are economically sustainable. This is also recognised in the sense of "molecular farming" in which genetically modified plants generate biomolecules of commercial value [3]. In the early 1990s, the idea of plant products acting as a distribution mechanism as well as biofactories made way for their use as oral vaccines. In order to beat the drawbacks of traditional vaccines, further experiments are under way to create edible formulations. Since the advent of the concept, the use of plants to manufacture vaccines has had many benefits. Plant vaccines reduce manufacturing costs and, as seen by the biopharmaceutical industry, make it easier to scale up. In molecular farming, the success of the Ebola drug ZMapp produced in tobacco plants resulted in the benefit of visibility. There is no need for pre-treatment or purification of edible vaccine formulations, adding further to the reduction in production costs. In addition, the presence of a rigid cell wall in plants guarantees the defense of antigen, known as the bio-encapsulation effect. Ultimately, since plant vaccines remove infection from mammalian-specific pathogens, plant-based oral vaccines offer improved protection relative to conventional ones. Long-term stability, higher antigen content and decreased microbial contamination are also given [3, 5].

Plants such as bananas, carrots, corn, tobacco, tomatoes and peanuts will gain appeal as EVs in the future with the active creation of genetic transformation techniques. In today's times, plant species such as carrot, papaya, potato, lettuce, tomato, quinoa and tobacco are used to convert into vaccine antigens. To date, few plant-based vaccines for hepatitis B virus (HBV), Norwalk virus, *Vibrio cholerae*, enterotoxigenic E. Coli, are in phase-I clinical trials and the virus of rabies. In the preclinical stages of development, few vaccines targeting pathogens such as avian influenza viruses (HPAI H5N1), coronaviruses and *Helicobacter pylori* remain. [5]

Algae-based edible vaccines

Green microalgae have been demonstrated to be highly helpful in the protein production process for a number of industrial and therapeutic applications. With an increased level of antigen expression, stably converted lines of green algae can be easily obtained. Unicellular green algae have all the beneficial properties of plants [5, 3]. This offers specific and numerous benefits over terrestrial plants, including terrestrial plants:

- Rapid accumulation of algal biomass.
- The entirety of the biomass accumulated can be used for the production of vaccines.
- Its development is independent of soil fertility.
- There are fewer chances of cross-contamination by adjacent food crops.
- Usage of enclosed bioreactors for its production enhances the biomass yield as well as reduces the environmental escapes issues.
- It can be efficiently lyophilized and can be kept at normal room temperature after drying.
- Its genetic modification is easy and hence, expression levels of foreign genes can be increased.
- It is a possible food source for a few animals, including human beings.

- It shows resistivity towards animal pathogens which makes them a good vaccine delivery system. [50]

The advantages consider algae to be a perfect host for vaccine delivery. At present, algae-based vaccines are in the clinical trial stage. In order to solve such problems such as low expressivity and lack of glycosylation after chloroplast expression, progressive work is underway on the preclinical specifics against Human Papilloma Virus (HPV), HBV and Foot and Mouth disease. Table 2 provides information about the diseases against which microalgae are being tested an edible vaccination method.

Table 2
Algal based edible vaccines for various diseases

Disease	Algal Host	Reference
Hepatitis B	*Dunaliella salina*	
	Phaeodactylum tricornutum	
Foot and Mouth Disease	*Chlamydomonas reinhardtii*	
Malaria	*Chlamydomonas reinhardtii*	[2]
Human Papilloma Virus	*Chlamydomonas reinhardtii*	
Classical Swine flu	*Chlamydomonas reinhardtii*	
Hypertension (angiotensin II)	*Chlamydomonas reinhardtii*	
White Spot Syndrome	*Chlamydomonas reinhardtii*	

Insect-cell based edible vaccines

Insect cell systems are widely adopted due to their ability to achieve co-translation and post-translational modification, as well as protein phosphorylation, glycosylation and protein treatment, and their high protein synthesis rate [52]. This expression system makes it possible to transform or transiently express cell lines driven by recombinant baculovirus infection. Due to rapid improvements, the expression mechanism of baculovirus-insect cells called BEVS is becoming one of the most widely known and accepted mechanisms as an alternative to recombinant protein production, including subunit vaccines [57]. Bombyx mori larvae or pupae are widely used in the industrial development of recombinant proteins and effective mechanisms for vaccine delivery. [59, 60]. It is called GRAS because baculovirus cannot reproduce in vertebrates. In addition, the bio capsule-like fat and protease inhibitors in the silkworm protect the recombinant protein from the enzyme activity in the gastrointestinal tract.

[Therefore, baculoviruses are considered healthy, non-pathogenic and non-infectious. Due to their safety and ability to effectively transduce mammalian cells, baculoviruses are also considered to be the delivery options for vaccine antigens. This BEVS/insect cell technology will not provide a place for biological contamination. In mammalian cell lines, recombinant baculovirus has an important gene therapy development mechanism [5]. There are currently three commercially available insect cell vaccines, namely Flublok for influenza, PROVENGE for prostate cancer and Cervarix for cervical cancer [58].

Whole-cell yeast based edible vaccines

On an industrial scale, yeast cells have been used for the development of heterologous proteins. Due to their ability to make post-translational changes, the GRAS status, and the cellular wall that protects the antigen around the gastrointestinal tract, engineered yeast cells build an enticing vaccine delivery system [13, 61] The main downside of this is hyper-glycosylation of recombinant proteins, but it has been solved by the generation of defective yeast N-glycosylation strains [62, 63]

Table 3
Status of development of whole yeast-based vaccines

Pathogen	Yeast host	Antigen	Clinical Trial Status	Indication
HBV	*Saccharomyces cerevisiae*	HBV(X/S/core)	Phase 2	Chronic HBV
HCV	*Saccharomyces cerevisiae*	HCV(NS3/core)	Phase 2	Chronic HCV

S: hepatitis B surface antigen, X: hepatitis B regulatory protein, NS3: hepatitis C non-structural protein

Whole-cell vaccines based on yeast have been intensively researched as a result of their ability to induce an immune response. Certain preclinical studies were performed on the basis of orally administered *Saccharomyces cerevisiae* for various infectious agents such as HPV and *Actinobacillus pleuropneumoniae*, the findings of which demonstrated the ability of this delivery mechanism to evoke a defensive mucosal and systemic immune response. This delivery system's enhanced immunogenicity is elucidated by the adjuvant activity of beta-glucans on the yeast cell wall. The immunomodulatory and adjuvant effects of binding innate pathogen receptors on macrophages, dendritic cells and neutrophils indicate this [56]. Two clinical trials have been developed at present. The first is GS-4774 for Hepatitis B Virus (HBV) treatment, and the second is GI-5005 for Hepatitis C Virus (HBV) treatment (HCV){table 3}. Good results in phase 1 were obtained in the first clinical trial for GS-4774. [56]. The findings did not seem to indicate a therapeutic advantage during phase 2 clinical trials performed in noncirrhotic, virally suppressed patients with chronic HBV infection. Both phases 1 and 2 showed encouraging and promising results in view of the clinical trials for GI-5005 [54]. GI-5005 was paired with Peg-IFN/ribavirin for use during this clinical trial.

Clinical Trials
Hepatitis B

Hepatitis B is one of the most prevalent human viral diseases. Based on the estimations of WHO, past or current HBV infections are evident in two million individuals around the world. Around 360 million people are persistently infected. Over 600,000 deaths occur from HBV related diseases such as hepatocellular carcinoma or liver cirrhosis. The cost of vaccine production and administration are the current limitations worldwide for the Hepatitis B vaccine. Plant-based

vaccines substitute the current Hepatitis B vaccines by lowering the production cost than the regular ones [21, 4]. Hepatitis B surface antigen (HBsAg) is used in the manufacture of an edible vaccine. It was the first antigen to be expressed in *Nicotiana tabacum*. The expression of a virus protein in the plant system was observed for the first time in that study. Initially, the amount of antigen expressed was 2-6ng/mg which was later increased to 66ng/mg with the usage of different promoter systems. Potatoes were preferred over tobacco in the manufacture of HbsAg for oral applications due to the high alkaloid content of tobacco. In the transgenic potato, the expression of HbsAg protein was observed in the vesicles of the endoplasmic reticulum using a transmission electron microscope (TEM) [43].

The quality of antigen produced in the plant is of utmost importance. Though there is no problem with raw consumption, humans cannot consume raw potato. Therefore, to compare the immune response elicited by cooked and uncooked potato, a thermal degradation study was conducted by boiling the transgenic potato. The groups vaccinated by cooked potatoes showed decreased antibody response. However, the risk of thermal degradation was reduced due to the encapsulation of HbsAg by the potato which stabilized the antigen [46, 25]. The criteria for an ideal hepatitis B vaccine are:

- The quantity of HbsAg (orally consumable amount).
- The concentration of antigen in the plant (to allow even dosing of subjects, concentration should be uniform)
- The plant should palatable so that it can be easily consumed.
- The HBsAg should be stable in the plant at ambient temperatures to enable prolonged storage [25].

Many studies are focused on the formulation of plant-derived vaccine tablets in lettuce against hepatitis B. When plasmid HbsAg subtype ayw was cloned into CaMv, it showed higher levels of expression in roots than leaf tissue of transgenic potato. In guarded greenhouses, tomatoes expressing HbsAg are being grown. It was shown that 30 tomato plants were enough to provide antigens for 4000 vaccines [5].

Measles

Every year, 800,000 people die from measles worldwide. It is a highly contagious disease that can cause pneumonia, encephalitis, and even death. Due to immunosenescence, the incidence of measles among middle-aged and elderly people in developed countries is on the rise. The traditional measles vaccine is a live attenuated vaccine which is unsuitable for oral administration and will decompose when exposed to heat. Therefore, cooling is a prerequisite for storage. The presence of maternal antibodies in live attenuated vaccines can reduce their effectiveness [5, 51, 18]. Measles Virus Hemagglutinin (MVH) was selected from Edmonston strain antigens to develop an edible vaccine that was transformed into tobacco plants using plasmids/vectors. It was observed that mice fed with tobacco plants expressed MVH-induced antibody serum. It can neutralize wild-type MV and maintain its immunogenicity. The results also showed the presence of secretory IgA antibodies in mouse stool samples [19-23]. The successful results

in mice led to similar experiments in primates. Genetically modified carrots have also been used to successfully obtain measles hemagglutinin, which triggers a neutralizing antibody response [24].The test animals took the genetically modified plantain orally to analyze whether there were specific anti-hemagglutinating antibodies in the serum samples. The results show that bananas can induce an immune response to antigenic hemagglutinin [25]. Transgenic lettuce, rice, and baby food against measles are under the developmental process. 35-50 grams of MV-H lettuce is sufficient to induce an immune response when administered together with CT-B (adjuvant), but a higher dose is required when administered alone [26].

Malaria

Malaria is a universal health concern caused by the genus *Plasmodium*. Globally, the significant cause of human mortality and morbidity is malaria. 300 to 500 million people get infected by malaria and result in the death of 1.5 to 2.7 million people. In recent years, the malaria situation worldwide has become notably worse due to the development of resistance by causative agents towards the control measures [18]. Malaria is an endemic disease in most of the sub-Saharan African countries and poses problems to countries such as Asia, Europe, the Middle East, and Latin America [27]. Humans get infected by *Plasmodium vivax* and *Plasmodium falciparum* resulting in malaria. Proteins of these organisms are the primary focus of research in the development of vaccines against malaria. The selection of the vaccine candidate antigen is very challenging because of the parasite's complex life cycle. The pre-erythrocytic stage of the parasite, asexual stages, and blood of the parasite are the major targets of several studies. Transgenic plants represent promising candidates in the development of malarial vaccines when considering poverty in the endemic areas of malaria [51]. In the development of edible vaccines, three antigens were selected, namely, Merozoite Surface Protein (MSP) 4 and 5 from *Plasmodium falciparum* and MSP4/5 from *Plasmodium yoelii*. Wang et al. have illustrated that oral immunization of recombinant MSP4/5, MSP1, and MSP4 in mice along with Cholera Toxin B (CTB) as a mucosal adjuvant, elicited antibody responses efficacious against the blood-stage parasite. Due to low antigen expression levels in plants, it has been suggested that a huge quantity of antigen should be administered to express the desired levels of immunity [6].

Cholera

In developing countries, ten million deaths occur annually due to cholera and other diarrheal diseases, predominantly among children [18]. Cholera is a bacterial disease ascribed to *Vibrio cholerae*, a Gram-negative bacteria. It results in acute watery diarrhea due to the colonization of this bacteria in the small intestine. It produces an enterotoxin called Cholera Toxin B [1gunasekharan]. The results of a study on CTB in plant expression system revealed its high immunogenicity as well as a high affinity towards intestinal epithelium cells as a carrier protein and adjuvant like LTB [28]. When CTB is orally administered, it acts as a potent mucosal immunogen. This is due to the binding of CTB to the eukaryotic cell surfaces through the GM1 ganglioside receptors located on the intestinal epithelium surface and hence, elicits a mucosal response towards the

pathogens. When it is coupled chemically with other antigens, its immune response is enhanced [2]. When transgenic potato containing the CT-B gene of *Vibrio cholerae* was fed to mice, it showed effective results by inducing intestinal and serum anti-CTB antibodies production. With periodic boosters, consuming one potato a week for one month shown to elicit enhanced immunity. Co-expression of LT-B and mutant cholera toxin subunit A (mCT-A) in crop seed showed efficacy through nasal administration and it was also extremely practical [17, 5]. Administration of transgenic rice expressing CTB to mice illustrated the neutralization effect on antibodies and conferred protection [51].

Norwalk Disease

Norwalk disease is a viral disease caused by the caliciviridae family member, Norwalk virus causing gastroenteritis in human beings. Insect cells expressing Norwalk virus capsid protein resulted in a protein that lacked viral RNA thus rendering it non-pathogenic [29]. The resulting particles were morphologically and antigenically similar to an authentic Norwalk virus [30]. Mason constructed pNV101, pNV102, and pNV140 plant expression vectors [31]. Through the freeze-thaw method, these plasmids were transformed by Agrobacterium tumifaciens. By using rabbit anti (i-rNV) serum diluted 1:10000 in 0.01M PBS, the Norwalk virus coat protein (NCVP) was quantified with ELISA [32]. From the plant tissue, the recombinant Norwalk virus-like particles were extracted and purified [31]. Through anion exchange chromatography, western blotting, and SDS PAGE, the purified protein was qualified as well as quantified. Production of mucosal and humoral antibodies was observed when mice were fed with recombinant protein [2]. The development of seroconversion was observed in 19 (95%) out of 20 people who were fed with the transgenic potato expressing Norwalk virus antigen. Genetically modified bananas and powdered tomatoes with the expression of Norwalk virus are underway to combat the Norwalk virus [17].

Anthrax

Anthrax is an infection caused by Bacillus anthracis. After the realization of Bacillus anthracis usage as a biological weapon, the development of a vaccine against anthrax gained more importance. Massive vaccinations of conventional anthrax vaccine imposed serious limitations which include its severe adverse effects and the requirement of frequent vaccinations. Hence, oral and nasal vaccines are required to elicit mucosal immune responses [33]. Biolistic transfer of pag gene (anthrax protective antigen- PA) into tobacco leaves expresses a protein structurally similar to the major protein in the existing vaccine. Edema factor and lethal factors were missing in this vaccine which was responsible for inducing potential toxic side effects. Inoculating transgenic spinach with TMV-expressing PA was suggested to be a safer vaccine. To produce Bacillus anthracis protective antigen in potatoes, Kim et al. used cholera toxin LTB as a carrier protein [28].

Foot and mouth disease

Foot and Mouth Disease (FMD) is a major livestock disease that is under control because of vaccinations. VP1 is the structural protein of the FMD Virus and

possess critical epitopes with the ability to produce antibodies [1gunashekaran]. Expression of highly immunogenic epitope VP1 of FMDV in alfalfa plants was demonstrated by Santos et al. (2002). Sera of intraperitoneally immunized mice were screened for anti-VP1 antibodies. The sera detected the presence of antibodies against native VP protein, synthetic peptide, and purified FMDV particles. Mice even survived after the challenge tests. This was the first example of a peptide-based vaccine that was produced in transgenic plants which ensured complete protection in the experimental hosts [34].

With the usage of the recombinant Tobacco Mosaic Virus (TMV), Wu et al. described the expression of FMDV serotype O in tobacco plants. FMDV particles obtained from tobacco plants were used to immunize guinea pigs. Out of eight guinea pigs orally fed with 3mg of tobacco plants, only three survived. A novel model for an oral FMDV vaccine was reported by Zhang et al. (2011). The two serotypes of FMDV namely O and Asia 1 were combined with VP protein. It was then transferred to E. coli with heat-labile enterotoxin B subunit (LTB) and maize with the genes of the Cholera toxin B subunit (CTB) as adjuvants. The expression of VP1 genes was analyzed in maize plantlets. It was the first study conducted on the combined expression of two serotypes of FMDV together as a potential feed additive [51]. Wang et al. used transgenic rice containing the capsid precursor polypeptide (P1) gene of FMDV with dual cauliflower mosaic virus (CaMV 35S) promoter. The expression level of P1 was 0.6-1.3 ug/mg of total soluble protein was seen especially in rice leaves [35].

Rabies

According to the WHO reports, annually 50000 deaths occur due to rabies which represents a severe threat to animals as well as humans. Generally, wild animals such as bats, foxes, and raccoons are the cause of the disease in the USA. Dogs are the vectors in the transmission of the disease to humans in countries such as Asia, South America, and Africa. For the production of the rabies vaccine, Alfalfa mosaic virus, rabies glycoprotein, and nucleoproteins were used. The transformation was done through the fusion of the antigenic gene with the viral envelope protein of the plant virus. Tobacco plants were transformed by the alfalfa mosaic virus genome that was genetically altered by the introduction of the T cell epitope of rabies nucleoprotein (31D) and B cell epitope of rabies glycoprotein (G5-24). The resulting recombinant protein was purified after two weeks. Immune responses were elicited in intraperitoneally immunized mice with the purified protein. For the expression of rabies virus proteins, Nicotiana benthamiana (wild type tobacco) and Spinacia oleracea (spinach) were also used. Human trials were initiated when intraperitoneally and orally immunized mice showed promising responses. During human trials, 150g spinach leaves/ dose were fed three times to the volunteers resulted in eliciting rabies specific IgA and IgG responses [51]. Transgenic tomato plants expressing the glycoprotein gene of rabies virus (ERA strain) using CaMV were able to elicit immune responses in animals [17].

Rotavirus Infection

Severe diarrhea in children is caused by human rotavirus. Rotavirus causes 6% of children deaths below 5 years old and 25% of diarrhea-related deaths in the

developing countries. Using potato, plant-based rotavirus vaccines were developed. Fusion product of Rotavirus VP7 glycoprotein with the endoplasmic targeting SEKDEL sequence was transformed by agrobacterium tumifaciens into a potato. Oral administration of potato tubers in mice resulted in the production of mucosal IgA and serum IgG responses against rotavirus VP7 glycoprotein [36]. Potatoes expressing full-length rotavirus VP7 gene were propagated for fifty generations and stable transcription of the VP7 gene was observed [37].

HIV

Initially, the splicing of HIV proteins into CaMV has been successfully achieved. CaMV as a promoter along with two HIV proteins were injected successfully into tomatoes and the resulting protein expressed was able to demonstrate through PCR in different plant parts as well as in the second-generation plant. For the expression of Tat protein cloned into TMV, it has been successfully inoculated into the spinach. 300-500ug of Tat antigen was found in each gram of spinach's leaf tissue. Oral administration of this spinach followed by DNA vaccinations in mice resulted in higher antibody titers than the controls [38, 39].

Human Papilloma Virus

6.1%of all cancer cases globally are caused by human papillomavirus (HPV). Out of them, 99.7% are the causatives of cervical cancer [40]. HPV16 is responsible for more than half of the cases [41]. The malignant cellular transformation involves the oncoprotein hr-HPV-E7 which is used as a perfect candidate in therapeutic vaccine development [42]. Demurtas et al. in his work mentioned the expressivity of attenuated HPV-E7 protein in the microalgae *Chlamydomonas reinhardtii* [44]. Positive results were seen in preclinical animal models. The biochemical and physical studies of this antigen are analyzed. New possibilities are open as this antigen expression is observed in algae [45].

Diabetes

Diabetes affects more than 100 million people globally. Type I diabetes is also called as Insulin Dependent Diabetes Mellitus (IDDM) and juvenile-onset diabetes. Children and young adults are primarily affected by it. It is an autoimmune disease in which the body's immune system destroys insulin-producing beta cells of the pancreas. Ma et al. in their research at the University of Western Ontario, demonstrated the prevention of diabetes in mice fed with transgenic plants producing diabetes-related protein. It was an idea based on oral tolerance property. Glutamic acid decarboxylase (GAD67) is a pancreatic protein linked to the onset of IDDM and it prevented diabetes when injected into mice. Transgenic tobacco and potato plants containing the gene for GAD67 was developed by the Canadian group. When these were fed to non-obese diabetic mice, they spontaneously developed insulin-dependent diabetes. Enhanced levels of IG1, an antibody associated with cytokines, were found in treated mice. Transgenic plants expressing the antigen retained immunogenicity as well as prevented diabetes in animal models. This is the first evidence for the usage of edible vaccines in the treatment of autoimmune diseases according to Ma [47, 48].

Bovine Pneumonia Pasteurellosis

In the development of an edible vaccine against Bovine Pneumonia Pasteurellosis, an antigen called Leukotoxin (LKt) from the bacterium Mannheimia haemolytica was selected. Its immunogenicity was retained through the removal of transmembrane domains of the bacterium. The complex formed by the fusion of LKt50 with a modified green fluorescent protein (mGFRs) is transcripted by CaMV 35S promoter and transformed into plants through Agrobacterium tumifaciens. Oral immunization of this edible vaccine showed positive responses in rabbits [49].

STDs

Oral administration of Human papillomavirus type-II (HPV-II) recombinant VLPs produced in insect cells to BALB/c mice elicits an immune response which is dependent on dosage and conformation, and genotype-restricted. Hence, for the prevention of anogenital HPV disease, VLPs may be effective oral immunogens [17].

Other applications
Cancer therapy

Plants can produce sufficient quantities of monoclonal antibodies for cancer therapy. As a vehicle for targeting doxorubicin for breast, ovarian, colon and lung tumors, Soybean has been genetically designed to produce monoclonal antibodies (BR-96). Non-Hodgkin's lymphoma is also under investigation [17].

Birth control

TMV has been genetically engineered to express a protein found in the zona pellucida (ZP3 protein) of mice. When this protein is injected, the antibodies produced could prevent the fertilization of eggs in mice [17].

The Green Vaccines

Plant chloroplasts are extensively being used for the manufacture of green vaccines. Chloroplast expression system has multiple advantages such as hyper expression of protein, oral delivery and transgene containment through maternal inheritance. Since the chloroplast genome is maternally inherited, in the subsequent chloroplast transformation, the protein would be absent in pollen, thereby lessening the transmission risk by cross-pollination. To date, 23 vaccine antigens have been expressed in chloroplasts against 16 different viral, bacterial and protozoan antigens. It includes HIV antigens p24, cholera, Rotavirus, anthrax, malaria, plague and amoebiasis [12 Sharma].

Role in autoimmune diseases

Due to unknown reasons, when auto-antigens are orally delivered can turn on suppressor cells of the immune system which suppress the immune activity and thus producing immunological tolerance. For example, when arthritis patients

consume collagen, it resulted in relief. This is commonly seen in test animals which is time as well dosage-dependent. Antigen has to be administered frequently in doses greater than immunogenic doses to induce tolerance. The autoimmune disorders which can be prevented are type I diabetes, multiple sclerosis, rheumatoid arthritis, graft rejection, etc. [53]. To suppress immune attack and to delay the onset of high blood sugar, potatoes expressing insulin and a protein called Glutamic acid Decarboxylase (GAD), linked to CT-B subunit were fed to mouse and the results were effective. Research work is under progress for multiple sclerosis, rheumatoid arthritis, and lupus and transplant rejection [17].

Recombinant proteins/drugs

Plants are genetically engineered not only for the production of antibodies and vaccines, but also for the production of enzymes, albumin, serum protease and interferons which are usually expensive to manufacture. Example, the production of Glucocerebrosidase (hGC) in tobacco plants for the treatment of Gaucher's disease. This development cuts off the cost of production by a thousand folds. The transgenic tobacco plant is being tested for the production of Interleukin-10 to treat Crohn's disease. For the large scale production of recombinant therapeutic proteins in plants, many industrial process have been developed. Many other compounds such as anti-viral protein which inhibits the HIV virus in vitro, angiotensin-I (antihypertensive drug) and trichosanthin (ribosome activator) are also being produced. Hirudin (an antithrombin) derived from the transgenic plant is now being produced at a commercial scale. Now, there is increasing usage of food crops in the production of pharmaceuticals and drugs [17].

Conclusion

Vaccination is the most effective strategy to reduce infectious diseases which represents a global concern. Unfortunately vaccines cannot prevent every disease. However, strategies such as the generation of neutralizing antibodies, antibiotics, and antiviral drugs have been developed against infectious agents. Currently, many other innovative approaches are under development. The major break-through in the branch of biotechnology is the discovery of edible vaccines and its wide acceptance and attention lead to its success. Edible vaccines trigger mucosal and systemic immune responses. When compared to conventional vaccines, edible vaccines do not require sophisticated equipment and machines for their production. Moreover, they are considered to be safe and are orally administered which further reduces the need for sterile injection conditions and storage facilities. These vaccines may lead to a safer future and more effective immunization. One of the problems with the edible vaccine is due to the notion that genetically modified crops are bad, which is prevailing in most of the developing countries. Genetically modified crops are getting better with the ever-evolving and growing technologies. Many diseases can be eradicated with edible vaccines popularized properly and distributed worldwide and thus saving millions of lives.

Acknowledgements

The authors would like to thank everyone who were involved directly and indirectly in successful completion of this work.

Significance of the study

The growth of the world's population and the continued emergence of diseases have led to the development of new, more effective vaccines against many diseases. Edible vaccines offer exciting opportunities to significantly reduce the burden of disease, especially in developing countries where vaccine storage and handling are a major problem as cold chain management is involved. Compared to other conventional vaccines, plant-based food vaccine technology is cost-effective, easy to use, can be stored and transported without refrigeration, is effective and safe, while promising a better preventive solution.

Financial support and sponsorship: Nil.
Declaration of competing interest : No conflict of interest is declared.

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