



STUDY OF ANAEROBES AND PARASITES ISOLATED FROM EDIBLE LEAFY GREENS

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ABSTRACT

Tulsi, Mint and Spinach all are leafy greens. They have tremendous medicinal properties thus they are used as culinary herbs or sometimes consumed as raw. Although sometimes they caused health issues due to presence of some enteropathogenic bacteria. In this study we are tried to identify anaerobic bacteria and protozoa through different types of laboratory examinations and also found them.

KEYWORDS: Anaerobes, protozoa, Enteropathogens, Tulsi, mint, spinach.

INTRODUCTION

Tulsi, mint and spinach all are herbs and very important as well as very beneficial for our health. However sometimes they contain some harmful bacteria and parasites that may cause foodborne infections. Tulsi is sometimes used as divine offering or Prasad. Not many studies have tried to find out the load of pathogenic bacteria, parasitic cysts and ova causing foodborne infections, in these leafy vegetables. In this study we have been tried to find the pathogenic microorganisms present on these herbs and their detrimental effects on the health of consumers.

❖ Tulsi

Holy basil (*Ocimum sanctum*) is commonly known as *Tulasi* in Sanskrit or *Tulsi* in Hindi.^[1] Holy basil is a perennial flowering plant from the family called *Lamiaceae*. While this plant is native to the Indian subcontinent, it may also grow throughout Southeast Asia.^[2] Tulsi has a special place in Ayurveda as well as the homes of Hindus in India. It is considered sacred by Hindus and worshipped by them and is also used medicinally to relieve symptoms like cough and cold. Three main types of Tulsi are seen growing in India

1. Bright green leaves called Ram Tulsi
2. Purplish green leaves called Krishna Tulsi
3. Common wild Vana Tulsi.

Tulsi has used as prasad or offering to God and also proved to be highly effective in protecting our body from various infections and diseases of the liver, skin, kidney, respiratory tract and other sites.

Tulsi is known to have many medicinal properties like:

- Antipyretic effect (relieves fever)
- It may also have anti-inflammatory activities
- It can act as an antiemetic (prevents vomiting)
- Helps lower the blood sugar (antidiabetic)
- It can as well act as a hypotensive agent (lowers blood pressure)
- It might also have hypolipidemic (lowers cholesterol) activity
- It might act as an analgesic (relieves pain)
- It might have anti-asthmatic activity
- It might be an hepatoprotective (liver-protective) agent
- It might help reduce stress (antistress)
- It might be a potent expectorant (expels mucous)
- It might have anticancer potential
- It might be a diaphoretic (induces sweating).

Owing to its potent health benefits, Tulsi is rightfully called the '**Queen of Herbs**'^[3]

However, in spite of having so many beneficial roles, Tulsi may harbour several harmful bacteria like-members of Order Enterobacterales (a previous large family of Gram-negative bacteria including a number of pathogens like *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp., *Shigella* spp., *Proteus* spp., *Serratia* spp. and other species), *Pseudomonas* spp and parasites like-*Cyclospora* spp.^[4,5]

❖ Pudina

Mint, scientifically termed as *Mentha spicata*, is an aromatic herb belonging to the family Lamiaceae. It is also called Spearmint in English and Pudina in Hindi. It is widely used in Indian kitchen for flavour. The herb originated in European countries.^[6] Fresh and dried plant and essential oils derived from Pudina are widely used in food, confectionery, cosmetic, chewing gum, toothpaste, and pharmaceutical industries.^[7] It is grown worldwide for commercial uses as a flavouring agent for food and to be used in medicines.^[8]

The health-promoting properties of the Pudina are given below

- It may act as carminative (for removal of gas from the stomach)
- It may exhibit antispasmodic property (used to relieve muscle spasms)
- It may have powerful diuretic activity (induces the kidneys to produce more urine output)
- It may have antibacterial property
- It may also have antifungal property
- It may show antioxidant effect (mitigates the damage caused by oxidants)^[7]
- It can show anti-diarrhoeal activity
- It may show anti-asthmatic activity
- It may help to reduce cold & fever
- It may help to manage weight
- It may act as an antidote (medication given to counteract a particular poison)^[8]

There is still no available information about the presence of any pathogenic bacteria or parasites in pudina/mint leaves, so have searched for them.

❖ Spinach

Spinach (*Spinacia oleracea*) is a green leafy flowering plant which is found mostly in central and western Asia. It belongs to the family Amaranthaceae. It is generally consumed either fresh, or after storage using preservation techniques by canning, freezing or dehydration. It may be eaten cooked or raw (young leaves), and the taste differs considerably. Its high oxalate content may be reduced by steaming.^[9]

The beneficial properties of spinach are listed below:

1. Promotes weight management (reduce weight gain risk by up to 82%)^[10]
2. Reduces cancer risk considerably^[11]
3. Protects eye health (being rich in Vitamins A & C which prevents age related degeneration)^[12,13]
4. Prevents hair loss (rich in iron)^[14]
5. Prevention of anaemia (rich in Vit. K & Iron)^[15,16,17]
6. High in antioxidants (scavenges free radicals and prevents oxidative stress).^[18]

Notwithstanding the fact that spinach is so beneficial for our health, still a variety of bacteria like *Salmonella* spp. *Shigella* spp. and others, and parasites like *Ascaris*

lumbricoides eggs, *Entamoeba histolytica* cysts, *Giardia lamblia* cysts may be present in it.^[19-25]

Tulsi, pudina and spinach are all used as food as well as for medicinal purposes. The harmful bacteria present in these edible leaves may serve as pathogens and they may be multi-drug resistant also.

MATERIALS AND METHODS

Time of study: Beginning of May 2023 to end of September 2023 (5 months).

Place of study: Department of Microbiology, BN campus, All India Institute of Hygiene and Public Health, Kolkata.

Type of study: Ours was a laboratory based observational study.

Sample size: One hundred and twenty samples (120) were collected from the markets of different zones in Kolkata (East, West, North & South) and if possible, from other places of West Bengal. Sample size was 120, calculated by method of convenience.

Methodology proper: Samples were collected in sterile universal containers or if packed, then in packets. Then samples were transported to the laboratory for testing. Samples was weighed and specific weight or volume was cultured on the following media

1. Mac Conkey agar with neutral red as pH indicator (Peptone, Neutral red, agar agar, Lactose, Sodium taurocholate, deionized water) for culturing aerobic LF or NLF bacteria.
2. Xylose Lysine Deoxycholate (XLD) media {Agar, Sucrose, Lactose, Sodium thiosulphate, L-Lysine, NaCl, xylose, Yeast extract, Sodium deoxycholate, Ferric ammonium citrate} for culturing aerobic bacteria like *Shigella* and *Salmonella* spp.
3. Robertson's cooked meat medium (RCM) {Cooked meat medium, Peptic digest of animal tissue, Dextrose, Sodium chloride, yeast extract, Iron filings, Hemin, Vitamin K} for culturing anaerobic bacteria. RCM was incubated for 2 days and then Gram stain was carried out to find anaerobic bacteria, by comparing growth with aerobic plates.
4. Selenite F broth was used for enrichment and later after 1 day of incubation at 37 degree C, it was subcultured from it on to XLD agar and MacConkey agar again. This was done to find *Shigella* spp. and *Salmonella* spp.
5. Normal saline and Lugol's Iodine has been used to detect the trophozoites and cysts of protozoa, and egg & larvae of helminths by making wet mount.

One gram of dry sample was mixed in 1 ml normal (0.9%) saline in a sterile universal container, vortexed and transferred to 10 ml test tube. Then it was centrifuged at 5000 rpm for 2 minutes, and with the deposit, saline mount, Lugol's Iodine mount and Gram

stain also done. Modified or cold ZN (Ziehl Neelsen)/ Kinyoun's stain was carried out with 4% Sulphuric acid (H_2SO_4) as decolorizer. Thus parasites were assessed by modified ZN stain and saline and Iodine mount. Also culture on the aforesaid media has been performed from

the deposit. The colonies of the bacteria that has been appeared on the culture media after overnight incubation, has been identified by Gram stain, Catalase, Oxidase and other standard biochemical tests.



Fig. 1: Image of ordinary Tulsi leaf. Source: author.



Fig. 2: image of Krishna/black Tulsi leaf. Source: internet.



Fig. 3: Spinach leaf. Source: <https://www.amazon.in/Fresh-Produce-Palak-Leaf-Bunch/dp/B01G6O0MQG>



Fig. 4: Mint/pudina leaf. Source: <https://www.goodhousekeeping.com/health/diet-nutrition/a47308/health-benefits-mint/>

RESULTS

Findings pertaining to anaerobes and protozoa only have been shown here. We found gram positive anaerobes with bulging spores and pathogenic parasites in many edible leafy greens.

➤ Anaerobes present in the edible leaves studied

a. Tulsi: - Most common were Anaerobic Gram-positive bacilli (GPB) without spore, followed by anaerobic Gram-positive cocci (GPC) and Anaerobic Gram-positive bacilli with bulging spore (suggestive of *Clostridium* spp). Some were *C. perfringens* as indicated by lecithinase test done on Egg yolk agar (incubated anaerobically) and Lactose fermentation in lactose broth, tested anaerobically.

b. Mint: - Most common anaerobes were Anaerobic Gram-positive cocci (GPC) without spore, followed by

anaerobic Gram-positive bacilli and Anaerobic Gram-positive bacilli (GPB) with bulging spore (*Clostridium* spp).

c. Spinach: - Most common were Anaerobic Gram-Positive bacilli (GPB) without spore, followed by anaerobic Gram-positive cocci (GPC) and Anaerobic Gram-positive bacilli with bulging spore (*Clostridium* spp).

These outcomes have also been shown below in Table 1.

➤ In this study we studied total 121 leaves of different types like –

Tulsi (*Ocimum sanctum*) – 43 specimens

Mint (*Mentha spicata*) – 40 specimens

Spinach (*Spinacia oleracea*) – 38 specimens

Table 1: Anaerobes present in the leaves.

Types of anaerobes	Tulsi (43)	Mint (40)	Spinach (38)
Gram Positive Cocci	12(27.90%)	15(37.5%)	16(42.10%)
Gram Positive Bacilli	13(30.23%)	22(55%)	25(65.79%)
Gram Positive Bacilli (with bulging spore) indicative of <i>Clostridium</i> spp.	3(6.98%)	1(2.5%)	4(10.52%)
Gram Negative Cocci	1(2.33%)	-	-

Parasites present in the edible leaves studied:

1. Tulsi: - Round undefined structures (8-10 μ m), *Cryptosporidium* spp., *Entamoeba histolytica* / *Entamoeba dispar*, *Entamoeba polecki* / *Entamoeba hartmannii*.
2. Mint: - *Cryptosporidium* spp.
3. Spinach: - *Cryptosporidium* spp.

Most of the parasites were found in the Tulsi leaves and rarely on the other leaves.

DISCUSSION

Green leafy vegetables are an excellent source of nutrients like Vitamins A, C, E, D, B Complex and iron. They are also a very good source of folate which is necessary for good heart health. These nutrients present in vegetables also have antioxidant effects which help to reduce the risk of cancer.^[26] However, they also may contain many pathogenic microorganisms like bacteria, oocysts of protozoa and ova of helminths. Sufficient fresh vegetable consumption is an essential dietary need for individuals worldwide. Due to the changes in dietary habits, the propensity to consume raw vegetables is on the rise. In particular, spinach is noted to be an excellent

source of many different nutrients, like phenolic compounds, beta carotene, vitamin C, and Potassium. However, fresh greens also allow the growth of various pathogenic bacteria, namely *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp., and *Shigella* spp., because of their wide surface area and potential for pathogen internalization in structures like the stomata. Additionally, in 2008, the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) ranked leafy green vegetables as the highest anxiety product group from microbial safety perspective.^[27] Furthermore, many outbreaks related to foodborne pathogens like *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* in fresh spinach have been reported recently.^[27] In 2006, multiple outbreaks of *E. coli* O157:H7 infection associated with contaminated baby spinach resulted in 205 confirmed cases of serious illnesses, 31 cases of haemolytic uremic syndrome (HUS), and three deaths. Hence if we consume these leafy vegetables raw or unprepared, it can spread many gut infections caused by bacteria, protozoa as well as helminths. These are of great public health importance. In a study from The USA, common bacteria retrieved from fresh cut spinach were *Pseudomonas fluorescens*. Equally important is the presence of anaerobes and protozoa in these edible leaves. Moreover, since gut protozoa like *Cryptosporidium* spp. can cause gastroenteritis in general public and these leaves can be had raw or semi raw, it poses a significant public health risk. Similar risk exists with *Clostridium* spp. (anaerobic Gram positive bacilli with bulging spore). As far as we know, such studies have not been done yet in this part of the country. More such studies are needed to explore this interesting area of public health.

REFERENCES

- Jamshidi N, Cohen MM. The clinical efficacy and safety of tulsi in humans: a systematic review of the literature (<https://doi.org/10.1155/2017/9217567>). Evid Based Complement Alternat Med. 2017; 2017: 9217567. doi:10.1155/2017/9217567.
- Petrusello M. Holy basil (<https://www.britannica.com/plant/holy-basil>). Encyclopædia Britannica
- <https://pharmeasy.in/blog/health-benefits-of-tulsi/>
- <https://ag.umass.edu/greenhouse-floriculture/photos/basil-pseudomonas#:~:text=Pseudomonas%20cichorii%20causes%20a%20bacterial,other%20ornamental%20and%20foliage%20plants.>
- <https://www.consumerreports.org/food-safety/basil-from-mexico-is-likely-cause-of-cyclospora-food-poisoning-outbreak/>
- Mentha spicata (Mint, Spearmint) | North Carolina Extension Gardener Plant Toolbox [Internet]. [cited 2022 Apr 7]. Available from: <https://plants.ces.ncsu.edu/plants/mentha-spicata/>
- Snoussi M, Noumi E, Trabelsi N, Flamini G, Papetti A, de Feo V. Mentha spicata Essential Oil: Chemical Composition, Antioxidant and Antibacterial Activities against Planktonic and Biofilm Cultures of Vibrio spp. Strains. Molecules [Internet], 2015 Aug 1 [cited 2022 Apr 7]; 20(8): 14402. Available from: <https://pubmed.ncbi.nlm.nih.gov/26262604/>
- Mahendran G, Verma SK, Rahman LU. The traditional uses, phytochemistry and pharmacology of spearmint (*Mentha spicata* L.): A review. Journal of Ethnopharmacology, 2021; 278: 114266. Available from: <https://pubmed.ncbi.nlm.nih.gov/34087400/>
- <https://en.m.wikipedia.org/wiki/Spinach>
- Nour M, Lutze SA, Grech A, Allman-Farinelli M. The relationship between vegetable intake and weight outcomes: A systematic review of cohort studies (<https://doi.org/10.3390/nu10111626>). Nutrients, 2018; 10(11). doi:10.3390/nu10111626.
- Vaňková K, Marková I, Jašprová J, et al. Chlorophyll-mediated changes in the redox status of pancreatic cancer cells are associated with its anticancer effects (<https://doi.org/10.1155/2018/4069167>). Oxid Med Cell Longev, 2018; 2018: 4069167. doi:10.1155/2018/4069167
- National Institutes of Health Office of Dietary Supplements. Vitamin A: Fact sheet for health professionals (<https://ods.od.nih.gov/factsheets/VitaminA-HealthProfessional/>).
- National Eye Institute. Age-related macular degeneration (<https://www.nei.nih.gov/learn-about-eye-health/eye-conditions-and-diseases/age-related-macular-degeneration>).
- Park SY, Na SY, Kim JH, Cho S, Lee JH. Iron plays a certain role in patterned hair loss (<https://doi.org/10.3346/jkms.2013.28.6.934>). J Korean Med Sci., 2013; 28(6): 934-8. doi: 10.3346/jkms.2013.28.6.934.
- Bai X, Long J, He X, et al. Overexpression of spinach non-symbiotic hemoglobin in (<https://doi.org/10.1038/srep26400>) Arabidopsis (<https://doi.org/10.1038/srep26400>) resulted in decreased NO content and lowered nitrate and other abiotic stresses tolerance (<https://doi.org/10.1038/srep26400>). Sci Rep., 2016; 6: 26400. doi:10.1038/srep26400.
- National Institutes of Health Office of Dietary Supplements. Iron: Fact sheet for health professionals (<https://ods.od.nih.gov/factsheets/Iron-HealthProfessional/>).
- National Institutes of Health Office of Dietary Supplements. Vitamin K: Fact sheet for health professionals (<https://ods.od.nih.gov/factsheets/VitaminK-HealthProfessional/>).
- Pizzino G, Irrera N, Cucinotta M, et al. Oxidative stress: harms and benefits for human health (<https://doi.org/10.1155/2017/8416763>). Oxid Med Cell Longev, 2017; 2017: 8416763. doi:10.1155/2017/8416763.

19. Abadias, M., Usall, J., Anguera, M., Solsona, C., and Vinas, I. Microbiological quality of fresh minimally processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology*, 2008; 123: 121-129.
20. Oliveira, M.A. Oliveira, V.M., Souza, A.M.M., Bergamini, E.C.P. Martini. Microbiological quality of ready-to-eat minimally processed vegetables consumed in Brazil, *Food Control*, 2011; 22(2011): 1400–1403
21. Tambekar, D.H, and Mundhada, R.H. Bacteriological quality of salad vegetable sold in Amravati city (India). *Journal of Biological Sciences*, 2006; 6: 28-30.
22. Al-Binali, A. M., Bello, C. S., El-Shewy, K., and Abdulla, S. E. The prevalence of parasites in commonly used leafy vegetables in South Western Saudi Arabia. *Saudi Medical Journal*, 2006; 27: 613–616.
23. Daryani, A., Ettehad, G. H., Sharif, M., Ghorbani, L., and Ziaei, H. Prevalence of intestinal parasites in vegetables consumed in Ardabil. Iran. *Food Control*, 2008; 19: 790–794.
24. Abougrain, A. K., Nahaisi, M.H., Madi, N.S., Saied M.M. and Ghenghesh, K.S. Parasitological contamination in salad vegetables in Tripoli-Libya. *Food Control*, 2010; 21: 760-762.
25. Fallah, A.A., Pirali-Kheirabadi, K., Shirvani. F. and Saei-Dehkordi. S.S. Prevalence of parasitic contamination in vegetables used for raw consumption in Shahrekord, Iran: Influence of season and washing procedure. *Food Control*, 2012; 25: 617-620.
26. Yan L. Dark Green Leafy Vegetables. <https://www.ars.usda.gov/plains-area/gfnd/gfhnrc/docs/news-2013/dark-green-leafy-vegetables#:~:text=Dark%20green%20leafy%20vegetables%20are,many%20of%20the%20B%2Dvitamins.last%20accessed%2009.5.23.>
27. Yoon-Jae J, Jae-Won H. Combined treatment of UV-A radiation and acetic acid to control foodborne pathogens on spinach and characterization of their synergistic bactericidal mechanisms. *Food Control*, 2019; 106: 106698. <https://doi.org/10.1016/j.foodcont.2019.06.024>.