

## POLYPHENOL-RICH SELECTED DIETARY FRUITS AND VEGETABLES INHIBIT ORAL MICROBIAL CONSORTIUM

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### Abstract

This study evaluated the effects of polyphenol-rich dietary fruits and vegetables on the oral microbial consortium of 20 volunteers. Methanol-ethanol extracts were prepared from the selected fruits and vegetables, and their antimicrobial activity was assessed against the prepared oral microbial consortium. *Emblica officinalis* fruit extract showed the largest inhibition zone, both the disc diffusion (17.7 mm) and agar well diffusion (18.4 mm) assays, followed by *Ipomoea aquatica* and *Enhydra fluctuans* at 2000 µg extract. *E. officinalis* and *I. aquatica* powders were also successively fractionated into *n*-hexane (Hex), chloroform (Chl), and finally methanol (Met) fractions. The strongest antimicrobial activity with 22 and 23 mm zone diameters was observed in the Chl fractions of *E. officinalis* and *I. aquatica*, respectively, followed by the Met fraction (19.6 mm) of *E. officinalis* at 1000 µg/well. The broth microdilution method revealed the minimum inhibitory concentration (MIC) of 4000 µg/ml for Chl and Met fractions. Therefore, *E. officinalis* and *I. aquatica* extracts, especially their Chl and Met fractions, can be incorporated as functional ingredients in mouthwash and toothpaste to manage oral microorganisms and associated hygiene.

### Introduction

Oral health is the health of the mouth that includes the teeth, gums, and the whole oral-facial system. Oral diseases include dental caries, tooth loss, periodontal disease, oral mucosal lesions, and oropharyngeal cancers, which affect 3.5 billion people worldwide. Among the diseases, dental caries and periodontal disease are the major dental diseases caused by various bacteria in the oral cavity (Selwitz *et al.* 2007). More than 750 species of bacteria have been reported in the oral cavity, and some of them are responsible for oral diseases (Jenkinson and Lamont 2005). The bacterial populations of *Streptococcus mutans*, *S. sobrinus*, *Lactobacillus* spp., *Enterococcus faecalis*, *Actinomyces* spp., and non-mutant Streptococci are involved in dental caries and also periodontal infections (Ruby *et al.* 2002, Wang *et al.* 2012). Furthermore, the oral cavity is a suitable habitat for diverse fungal communities, including 101 species of fungi, of which the most dominant genus is *Candida*, followed by *Cladosporium*, *Aureobasidium*, Saccharomycetales, *Aspergillus*, *Fusarium*, and *Cryptococcus* (Ghannoum *et al.* 2010). Various species of *Candida* are responsible for oral candidiasis (Hu *et al.* 2019) and also for periodontal diseases (Unniachan *et al.* 2020).

Although various antibiotics are effective in preventing dental caries and periodontitis, they show side effects such as microbial resistance, nausea, hypersensitivity reactions, vomiting, hepatotoxicity, diarrhea, and allergic disorders (Ahmadi *et al.* 2021). Moreover, the use of the mouthrinse- chlorhexidine causes unpleasant taste sensations, tongue discoloration, and oral lesions (McCoy *et al.* 2008). Therefore, it is essential to explore new antimicrobial components from natural resources for controlling oral microorganisms. Reports showed that natural products are effective against gingivitis and bacterial plaque when used in mouthwash and toothpaste (Sener and Kilic 2019, Nordin *et al.* 2020).

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Both the direct and indirect costs of oral diseases were estimated at \$356.80 and 187.61 billion in 2015 (Peres *et al.* 2019). In developing countries such as Bangladesh, more than 80% of the population suffers from one or more dental diseases (Banglapedia 2015), and they can meagerly afford the modern treatment costs. Therefore, it is essential to find effective, low-cost alternatives for controlling oral diseases. A recent global trend is to find preparations rich in phytochemicals, especially polyphenols, to combat infectious microorganisms. Though different types of Bangladeshi dietary fruits and vegetables (Hossain *et al.* 2008, 2014, 2015, Alam *et al.* 2021, Hosen *et al.* 2021, Biswas *et al.* 2023) showed potent health benefits, none of them were reported to be used for controlling oral microorganisms, and therefore, the study was undertaken. The findings of this research offer scientific support to the use of dietary fruits and vegetables as an alternative for the prevention and treatment of oral diseases.

### Materials and Methods

Based on previous studies (Hossain *et al.* 2014, 2015, Alam *et al.* 2021), eleven dietary fruits and vegetables rich in polyphenols, including *Abelmoschus esculentus* (L) Moench. fruit (okra, lady's fingers); *Basella alba* (L) stem and leaves (*Malabar spinach*); *Emblica officinalis* (L) Gaertn. fruit (amla, Indian gooseberry); *Enhydra fluctuans* (Lour) stem and leaves (helencha, buffalo spinach); *Ficus hispida* (L) fruit (hairy fig); *Ipomoea aquatica* (Forssk) stem and leaves (water spinach); *Lablab purpureus* (L). fruit (lablab bean); *Lagenaria siceraria* (Molina) Standl. Santol fruit (bottle gourd); *Moringa oleifera* (Lam) fruit (drumstick); *Psidium guajava* (L) fruit (guava); and *Solanum melongena* (L) fruit (eggplant; brinjal) were collected from different local markets in Khulna City, Bangladesh. The fruits and vegetables were thoroughly washed in distilled water. Then they were cut into small pieces and shade-dried. Using a grinding machine, the dried fruits and vegetables were ground into fine powders and stored separately in airtight containers at room temperature.

Fine powder (20 grams) of each fruit and vegetable was taken in separate extraction bottles. Then, the powder of each bottle was mixed thoroughly with a 200 mL methanol-ethanol (1:1) mixture. The mixtures were occasionally shaken for 3 days at room temperature, and then filtered using filter paper (Whatman No. 1). After drying in air, the filtrates turned into thick extracts, which were weighed and stored at 4°C in a refrigerator. The yield of each extract was calculated.

Fine powder of the potential fruits and vegetables was successively fractionated into *n*-hexane (Hex), chloroform (Chl), and methanol (Met) fractions. In brief, 20 g of powder was fractionated in 200 ml of *n*-hexane for 3 days at room temperature with occasional shaking. Hereafter, the fraction was collected through filtration using Whatman No. 1 filter paper. The filtrate was then dried at room temperature, weighed, and labeled as an *n*-hexane fraction (Hex). Following the same method, the residue on the filter paper was then used for preparing chloroform (Chl) and finally methanol (Met) fractions. All the fractions were stored at 4°C in a refrigerator until the experiments.

An oral microbial consortium was prepared by swabbing the teeth, gums, saliva, and tongue surfaces of 20 volunteers with sterile cotton buds. The microorganisms attached to the cotton buds were then inoculated into the nutrient broth medium (NB) supplemented with yeast extract, and incubated at 37°C for 24 hrs. After the growth, the microbial consortium was preserved at 4°C in a refrigerator. For long-term preservation, the freshly prepared consortium was transferred into one-drum vials and then stored at -22°C, aided with 15% sterile glycerin.

For the disc diffusion test, 100 µl of freshly prepared oral microbial consortium (OD 0.2 at 600 nm) was uniformly spread on the top of the solidified nutrient agar (NA) medium in Petri plates. Then, sterile paper discs (6 mm) impregnated with different concentrations of extract were

placed on the surface of the medium, and left for 30 min at room temperature for diffusion. Discs impregnated with methanol-ethanol and tetracycline served as the control (C) and positive control (PC, 20 and 200 µg/disc), respectively. After incubation at 37°C for 24 hrs, the inhibition zones were recorded. Each experiment was replicated three times.

For the agar-well diffusion assay, a 24 hrs broth culture of the oral microbial consortium (OD 0.2 at 600 nm) was spread on NA medium with a sterile glass rod. Wells (10 mm diameter) were made at proportionate distances on the NA medium using a sterilized cork-borer. Extracts, fractions, methanol-ethanol (1:1, control, C), and tetracycline (positive control, PC, 20 and 200 µg/well) were transferred into the wells, and incubated at 37°C for 24 hrs. The diameter (mm) of the inhibition zones around the wells was recorded. Three replicates were performed for each experiment.

The broth microdilution method was followed to determine minimum inhibitory concentration (MIC) using a sterile 96-well microtitre plate. Nutrient broth mixed with either Met or Chl was transferred into the wells and inoculated with the microbial consortium. After mixing, the plate was incubated at 37°C for 24 hrs. The MIC was determined by observing the lowest concentration of the fraction that inhibited the growth of the consortium. Resazurin was used to assess respiratory activity in the wells. Tetracycline was used as a positive control.

The results were expressed as mean  $\pm$  SD (standard deviation), where  $n = 3$ . Data were analyzed using one-way ANOVA, followed by multiple comparison Tukey's test. Statistical significance was set at  $p < 0.05$ .

## Results and Discussion

Among the selected fruits and vegetables rich in polyphenols, *E. officinalis* showed the highest extract yield (35%) extracted with methanol-ethanol followed by *S. melongena* (22%), *L. siceraria* (19%), *B. alba* (19%), and *P. guajava* (17%), whereas, the lowest yield (7.8%) was observed from *A. esculentus* (Fig. 1). The extract yield differences of these fruits and vegetables were probably dependent on their molecular composition, the extraction conditions, and the solvent used. The conditions and solvents given in this experiment effectively extracted the components from *E. officinalis* compared to *A. esculentus* fruits. Romdhane *et al.* (2020) reported a high content of insoluble fiber in the fruit of *A. esculentus*.

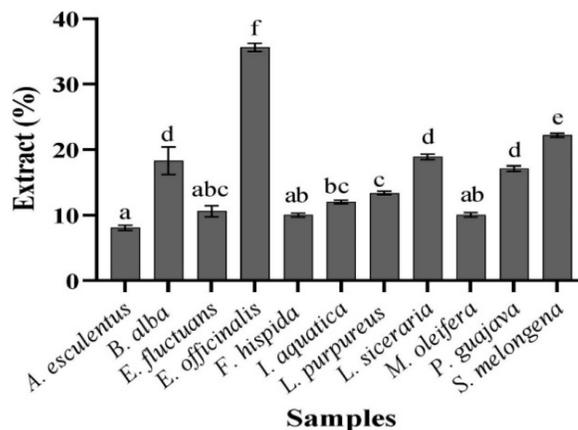


Fig. 1. Extracts were obtained using methanol-ethanol (1:1) for the extraction of selected polyphenol-rich dietary fruits and vegetables. Data were presented as mean  $\pm$  SD, with  $n = 3$  observations. Different letters indicated significant differences at  $p < 0.05$ .

The disc diffusion assay was conducted using the extract concentrations of 1000, 1500, and 2000  $\mu\text{g}/\text{disc}$ . All the extracts at the concentration of 2000  $\mu\text{g}/\text{disc}$  significantly ( $p < 0.05$ ) inhibited the oral microbial consortium (Fig. 2). *E. officinalis* extract showed the highest inhibition zone of 17.8 mm at 2000  $\mu\text{g}/\text{disc}$ , which was followed by *E. fluctuans* (16.7 mm), *I. aquatica* (14.6 mm), and *L. purpurea* (13.3 mm). The agar well diffusion assay was also performed with the extracts. It also showed significant ( $p < 0.05$ ) inhibition of the oral microbial consortium at the concentration of 2000  $\mu\text{g}/\text{well}$  (Fig. 3). Among the extracts, *E. officinalis* showed the strongest antimicrobial effect with an inhibition zone of 18.4 mm, which was followed by *I. aquatica* (17.2 mm) at 2000  $\mu\text{g}/\text{well}$ . Alam *et al.* (2021) reported that among Bangladeshi fruits, *E. officinalis* contains the highest amount of polyphenols (89 mg/g powder). In leafy vegetables, *I. aquatica* is composed of the largest amount of polyphenols (38.9 mg GAE/g extract) (Hossain *et al.* 2015). It was reported that polyphenol-rich food extracts inhibited the growth of *Streptococcus mutans*, a causative bacterium for dental caries, and other oral pathogens (Smullen *et al.* 2007). *E. officinalis* fruit extract also showed inhibition of growth and aggregation of halitosis-related bacteria, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Solobacterium moorei* (Lu *et al.* 2024). Arif *et al.* (2014) reported the strongest antibacterial activity among antidiabetic medicinal plants through the presence of high polyphenols.

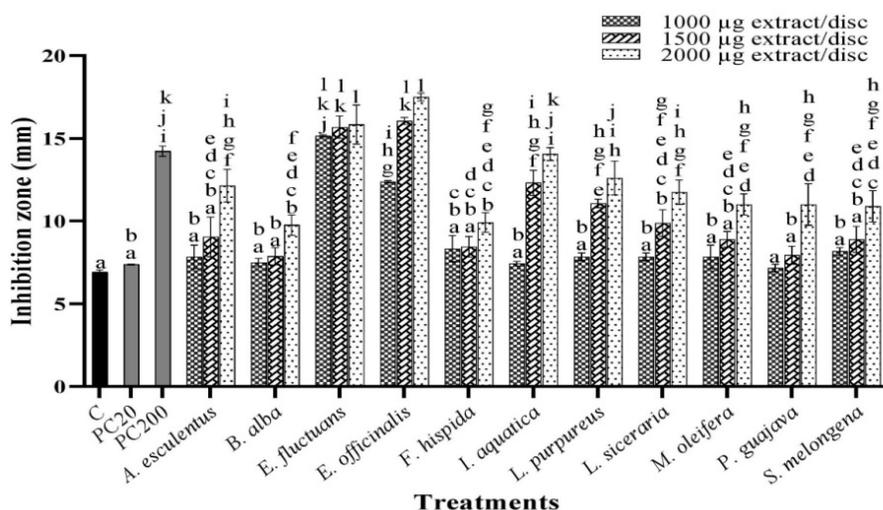


Fig. 2. Inhibitory effects of extracts of selected fruits and vegetables on oral microbial consortium by disc diffusion. Different letters (a-l) indicated significant differences at  $p < 0.05$ .

From the disc diffusion and agar well diffusion assays, *E. officinalis* fruit extract was the most potent in inhibiting the oral microbial consortium, followed by *I. aquatica* and *E. fluctuans*. Among the three plants, the fruits of *E. officinalis* and the stem and leaves of *I. aquatica* are consumed popularly, and therefore, selected for successive fractionation, starting from *n*-hexane (Hex), then chloroform (Chl), and finally methanol (Met). The antimicrobial activity of the fractions against the oral microbial consortium was assayed using agar well diffusion. The highest inhibition zones were observed from Chl fractions of *I. aquatica* (22.9 mm) and *E. officinalis* (22.1 mm) at 1000  $\mu\text{g}/\text{well}$ . It was followed by the Met (19.6 mm) fraction of *E. officinalis*, and the Hex (15.8 mm) fraction of *I. aquatica* (Fig. 4). The MICs of the Chl and the Met fractions of *E. officinalis* and only the Chl fraction of *I. aquatica* were determined. Wells containing the

concentrations of 125, 250, 500, 1000, 2000, 4000, and 6000  $\mu\text{g/ml}$  fractions were inoculated with the same volume of freshly prepared consortium. It was observed that as the fraction concentration increased, the growth (OD, 600 nm) of the oral microbial consortium decreased. All the fractions showed the MIC of 4000  $\mu\text{g/ml}$ . Hasan *et al.* (2012) reported that crude extract and ethanol fraction of *E. officinalis* fruit reduced adherence, biofilm formation, and glucan synthesis, and suppressed virulence genes of *Streptococcus mutans*. Weiss *et al.* (2002) reported that a cranberry constituent dissociated co-aggregation of oral inter-generic bacteria, and reduced *Streptococcus mutans* counts in saliva.

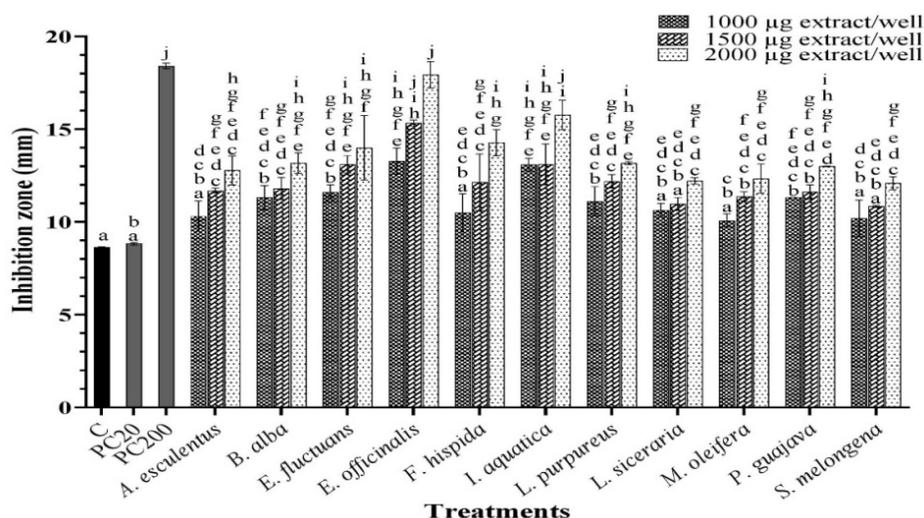


Fig. 3. Inhibitory effects of methanol-ethanol extracts of selected fruits and vegetables on oral microbial consortium by agar well diffusion. Different letters (a-j) indicated significant differences at  $p < 0.05$ .

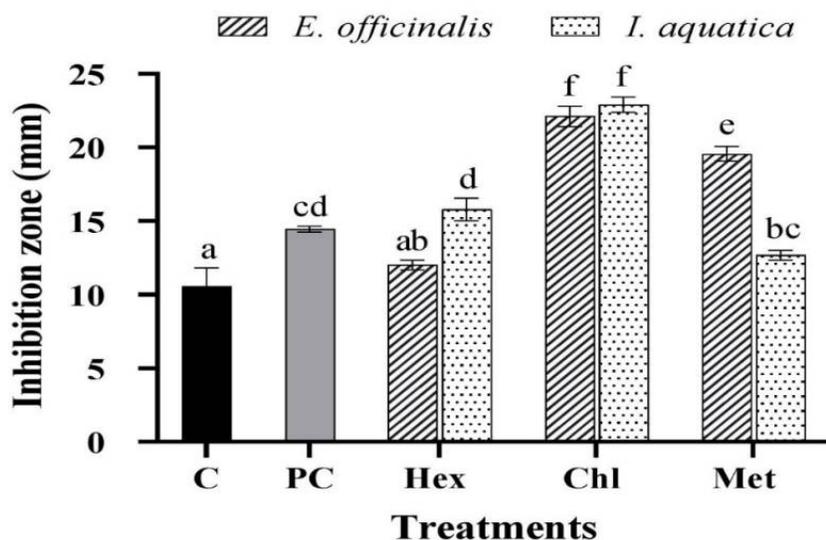


Fig. 4. Inhibitory effects of the fractions of *Emblica officinalis* and *Ipomoea aquatica* on oral microbial consortium by agar well diffusion at 1000  $\mu\text{g}$ /well. Different letters (a-f) indicated significant differences at  $p < 0.05$ .

The results suggested that among polyphenol-rich dietary fruits and vegetables, *E. officinalis* fruit inhibited the oral microbial consortium strongly, followed by *I. aquatica* and *E. fluctuans*. Therefore, they can be incorporated as a functional supplement in the preparation of toothpaste, mouthwash, and similar products to maintain oral hygiene. It is also essential to find the active compound(s) from *E. officinalis*, *I. aquatica*, and *E. fluctuans* to fight oral ailments.

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