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ORIGINAL ARTICLE

Antimicrobial Activity of Soyabean (*Glycine max*) and Pumpkin Seeds (*Cucurbita pepo*) Extracts against Common Oral Pathogens: An *in vitro* Study

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ABSTRACT
Objective: To evaluate the antimicrobial activity of soyabean and pumpkin seeds methanolic extract against oral microbiome. **Material and Methods:** An *in vitro* study compared the antimicrobial activity of methanolic extract of soyabean and pumpkin seed along with 0.2% chlorhexidine (positive control) by Agar well diffusion method against *Streptococcus mutans*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Lactobacillus* and *Candida albicans*. Suitable dilutions of each seed extract determined Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Qualitative phytochemical analysis and Thin Layer Chromatography (TLC) was done to determine the phytochemical components. **Results:** Both the methanolic extracts showed the highest zone of inhibition, 26±0.31 mm and 24±0.82 mm, followed by 22±0.87 mm and 21±0.55 mm against *Streptococcus mutans* and *Streptococcus pyogenes*, respectively. The zone of inhibition was higher at concentrations in both extracts, which was not statistically significant ($p>0.05$). MIC values fluctuated from 1.56 to 4.5 mg/ml and 3.5 to 5.0 mg/ml for soyabean and pumpkin seed extract, respectively. Phytochemical analysis showed the presence of alkaloids, glycosides, terpenoids, tannins, and flavonoids. **Conclusion:** Both extracts were effective against *S. mutans* and *S. pyogenes* and contain compounds with therapeutic potential.

Keywords: Microbial Sensitivity Tests; Cucurbita; *Streptococcus mutans*.

Introduction

Oral diseases constitute one of the significant burdens on the health sector of many countries [1]. It affects people throughout their lives, causing pain, discomfort, disfigurement, and even death. Oral health even affects a person's overall health and shares a common risk factor. The Global Burden of Diseases 2017 has stated that dental caries in permanent teeth are the most common health condition. Oral health treatment is costly and relatively inaccessible in many developing countries as they need the required infrastructure for establishing a dental clinic.

Though the world around us is developing profusely in the field of medicine, a lot of portions are still left untouched. The pharmacological industries have delivered a variety of new antibiotics over the decades, and the microbes have also evolved and developed resistance to the antibiotics. The oral cavity is a niche for different kinds of bacteria and fungi. It's essential to check their growth. Reports have revealed that these microbes have developed microbial resistance to the antibiotics commonly used to treat oral infections, like penicillin, cephalosporins, erythromycin, tetracycline and derivatives, and metronidazole [2]. To treat such organisms, it is important to explore the world of medicinal plants and their products for antimicrobial properties.

The Soyabean, soybean, or soya bean (*Glycine max*) is a legume species native to East Asia. It is widely grown for its edible beans, which have numerous uses. Soyabeans contain a significant amount of phytic acid, dietary minerals, and vitamin B complex. The soyabean plant is one of the important sources of high protein and lipid content and other major constituents, including vitamins, minerals, fatty acids, and other essential nutritional factors. There is evidence regarding the good effects of soyabeans on cancer and cardiovascular health. Soyabeans are of much importance from a medicinal perspective. Recorded data revealed the high antimicrobial and antioxidant content of the *G. max* [3,4].

Similarly, Pumpkin (*Cucurbita pepo*), native to North America, is one of the oldest domesticated plants, having been used as early as 7000 to 5500 BC. Pumpkins are widely grown for commercial use, food, aesthetics, and recreational purposes. Pumpkin is an excellent source of provitamin A, beta carotene, and vitamin A; vitamin C is present in moderate content. Pumpkin seeds, also known as pepitas, are edible and nutrient-rich. These are a good source of protein, magnesium, copper, and zinc [5,6].

Even though there are studies regarding the antibacterial effect of soyabean and pumpkin seeds, more concrete evidence is required, the present study was done to assess the antimicrobial effects of the methanolic extract of the soyabean and pumpkin seeds against oral microflora.

Material and Methods

Ethical Issues

The Ethical Council of the Institute approved the study (Ref No. SOA/IDS/2021/18).

Isolation of Bacterial Isolates

Test microorganisms were collected from the Central Research Laboratory, Institute of Dental Sciences, Bhubaneswar. Four bacteria, including *Streptococcus mutans*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Lactobacillus*, along with one fungus, *Candida albicans*, were used.

Preparation of Seed Materials

The freshly collected dried seed samples were cleaned with tap water and were then again shed-dried. Further, they were crushed to powders using a mixer grinder.

Preparation of Seed Extracts

For extraction, a 40 g lot of powdered seed material was dissolved in a volume of 400 ml (approximately) of an organic solvent (methanol) in a screw cap jar. The mixture was stored for 7 days at 4°C.

Agar-Well Diffusion Method for Antibacterial Assays of Seed Extracts

Antibacterial activity of the methanol extracts of the seed sample was done by the agar-well diffusion method. Microbial lawns were prepared with agar 6 mm thick that was fully punched, and 6-8 wells were prepared when a lawn was 30 minutes old, and each well was based on 50 µl molten Mueller-Hinton agar in duplicates. Further, wells were filled with 100 µl aliquots of 0, 25, 50, 75, and 100 mg/ml solvent-extracts of the seed, diluted from the original stock of seed extracts of individual organic solvents, by 10% DMSO solution. Plates were incubated at 37°C for 18-24 hours. Antibacterial activities were evaluated by measuring the diameter values of zones of inhibition. An experiment of each solvent extract was conducted thrice, and data from the third repeated experiment were presented. An aliquot of 100 µl of 2% chlorhexidine with an average diameter of zone of inhibition of 20 and 18 mm and 10% DMSO solution was the reference control [7], and 10% DMSO solution had no antibacterial activity [8].

Determinations of MIC and MBC Values of the Seed Extracts

MIC and MBC of active seed extracts, prepared with methanol, were determined by suitable dilutions from original stock solutions of each seed extract for concentrations 0, 25, 50, 75, and 100 mg seed-extract/ml in aliquots of 10% DMSO solution. Separate experiments were conducted for each solvent extract. An aliquot of 80 µl of each dilution of a solvent-extract was released to a well on a 96-welled (12×8) microtiter plate, along with an aliquot of 100 µl MH broth (HiMedia), an aliquot of 20 µl bacterial inoculum (109 CFU/ml) and a 5 µl-aliquot of 0.5% TTC. After pouring all the above materials into a well, the microtitre plate was incubated at 37°C for 18 hours. The development of pink colouration due to TLC in a well-indicated bacterial growth and the absence of the colouration was taken as growth inhibition. The first well of the microtitre plate was the control, without any seed extract [9]. The MIC value was noted at the well, where the pink colour was not manifested. Further, bacteria from each well of the microtitre plate were sub-cultured on nutrient agar; the level of dilution, where no bacterial growth on the nutrient agar was observed, was noted as the MBC value. Results of the second repeated experiment were presented [8].

Qualitative Phytochemical Analyses

- Test for reducing sugars: An Aliquot of 2 ml of the seed extract in a test tube was mixed with a 5 ml mixture of equal volumes of Fehling's solutions I and II, and the mixture was heated in a water bath for about 2 minutes. A brick-red precipitate between the aqueous extract and Fehling solution I and II indicated the presence of reducing sugars.
- Test for saponins: A lot of 0.5 g of the seed extract was dissolved in an aliquot of 10 ml of distilled water in a test tube, which was shaken vigorously for 30 seconds and subsequently allowed to stand for 45 minutes. The appearance of frothing on warming the lot indicated the presence of saponins.
- Test for flavonoids: To an aliquot of 0.5 ml of the dissolved seed extract, a few drops of 10% ferric chloride solution were added. A blue-green coloration indicated the presence of flavonoids.
- Test for tannins: A lot of 0.5 g of the seed extract was dissolved in a 5 ml aliquot of water, followed by the addition of a few drops of 10% ferric chloride solution. A blue-black, green, or blue-green precipitate indicated the presence of tannins.

- Test for steroids/terpenes: A lot of 500 mg of the concentrated mass of the seed extract from the rotary evaporator was dissolved in an aliquot of 2 ml of acetic anhydride, and the mixture was cooled at 0 to 4°C, to which a few drops of 12N sulphuric acid were carefully added. A color change of the solution from violet to blue-green indicated the presence of a steroidal nucleus.
- Test for alkaloids: A lot of 0.5 g of seed extract was stirred with an aliquot of 5 ml 1% HCl on a steam bath, and the mixture was filtrated to an aliquot of 1 ml of the filtrate, a few drops of Mayer's reagent (1.36 g $HgCl_2$, 5 g KI in 100 ml distilled water) was added, and to another aliquot of 1 ml of the filtrate, a few drops of Dragendorff's reagent (two solutions in 1:1 ratio 'solution A' with 0.85 g bismuth nitrate, 10 ml glacial acetic acid and 40 ml distilled water, and 'solution B' with 8 g KI in 30 ml distilled water) were added. Turbidity or precipitation in tubes due to either of these reagents indicated the presence of alkaloids in the seed extract.
- Test for glycosides: An aliquot of 5 ml of seed extract was mixed with an aliquot of 2 ml of glacial acetic acid (1.048 g/ml), one drop of 1% $FeCl_3$ solution, and mixed thoroughly to which an aliquot of 1ml of 12N H_2SO_4 was added. A brown ring at the interface indicated the presence of glycosides. All these tests were repeated for confirmation [8].

Thin Layer Chromatography of Seed Extract

Thin-layer chromatography results confirmed the presence of different bioactive compounds. TLC plates were prepared by using silica gel as an adsorbent. 10mg of silica gel was mixed with 23 ml of distilled water to make a slurry. The slurry was immediately poured uniformly onto glass plates. The plates were then allowed to air dry. The layer was fixed by drying at 55ºC for one hour. The mobile phase was prepared using solvents like toluene, acetone, and formic acid in a ratio of 4.5:4.5:1 to separate the metabolites. The chamber was closed for 1 hour for supersaturation. About 10 µL of extracts were loaded gradually over the plate and air-dried using a micropipette. The plates were placed in a TLC chamber and covered, ensuring that the solvent was just below the spots. The plates were removed when the solvent moved close to the top edge, marked as the distance travelled by the solvent. The spots moved by the solvent were marked, and the shape of the spots was also marked. The distance moved by the solvent and spots were measured in centimetres. Flavonoids are detected in visible light and by spraying aluminium chloride. The Rf value was calculated. The retention factor, Rf, is defined as the distance travelled by the compound divided by the distance travelled by the solvent.

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front TLC plates}}$$

Results

The observations of antimicrobial properties of methanolic extract of soyabean seed and pumpkin seed and the positive control 2% Chlorhexidine are presented in Table 1. The agar well diffusion method showed the largest zones of inhibition of the methanolic soyabean and pumpkin seed extract to *Streptococcus mutans* (26±0.31 mm and 24±0.82 mm) and *Streptococcus pyogenes* (22±0.87 mm and 21±0.55 mm) respectively at a concentration of 100 mg/ml. When compared with the control, both seed extracts demonstrated a lower zone of inhibition against *S. aureus*, *Lactobacillus*, and *Candida albicans*. However, the differences observed in the extracts concerning the zone of inhibition were not statistically significant ($p>0.05$) at all four concentrations against the five microbes. The extract showed high activity against *S. mutans* and *S. pyogenes* species but didn't possess antifungal properties.

Table 1. An antimicrobial assay is a zone of inhibition of methanolic seed extract of Soyabean and pumpkin seed extract using the agar well diffusion method.

Bacteria	Zone of Inhibition (mm)								
	Soyabean Seed Extract ([ ] mg/ml)				Pumpkin Seed Extract ([ ] mg/ml)				Positive Control
	25	50	75	100	25	50	75	100	
S. mutans	23±0.12	23±0.36	24±0.58	26±0.31	21±0.32	22±0.22	23±0.13	24±0.82	22±0.13
S. pyogenes	17±0.82	19±0.32	21±0.92	22±0.87	16±0.61	17±0.45	19±0.42	21±0.55	10±0.25
S. aureus	20±0.76	20±0.61	21±0.48	21±0.36	20±0.21	19±0.15	20±0.41	21±0.34	22±0.32
Lactobacillus	19±0.39	19±0.43	20±0.42	21±0.29	18±0.47	19±0.75	20±0.28	20±0.62	21±0.16
Candida albicans	17±0.27	17±0.12	18±0.08	18±0.43	19±0.14	20±0.21	20±0.52	21±0.71	23±0.23
F-value	1.6552				1.2825				
p-value*	0.2165				0.3142				

*One-way ANOVA.

Further, both methanolic extracts were subjected to determine the MIC and MBC values. Out of the two seed extracts, the soyabean seed extract showed antimicrobial action with MIC values fluctuating from 1.56-4.5 mg/ml against oral pathogens, and pumpkin seed extracts showed antimicrobial action with MIC values fluctuating from 3.5-5.0 mg/ml against oral pathogens (Table 2). The MBC values were recorded as 11.5-15.0 mg/ml for soyabean seed extracts and 12.5-17.0 mg/ml for pumpkin seed extract.

Table 2. MIC and MBC values of two seed extracts against oral microflora.

Bacteria	Methanolic Seed Extracts			
	Soyabean Seed		Pumpkin Seed	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
S. mutans	1.56	11.5	3.5	11.5
S. pyogenes	3.0	11.5	4.5	15.0
S. aureus	3.5	11.5	3.0	12.5
Lactobacillus	4.5	15.0	5.0	17
Candida albicans	1.56	11.5	3.5	15.0

Standard procedures for preliminary phytochemical screenings of the extracts were carried out to analyze the presence of various constituents. The qualitative phytochemical analysis of the methanolic soyabean seed extract revealed the presence of alkaloids, glycosides, terpenoids, flavonoids, reducing sugars, tannins, and steroids, but saponins were absent (Table 3). In pumpkin seed extract, alkaloids, glycosides, terpenoids, flavonoids, tannins, and saponins were confirmed, but reducing sugars and steroids were absent. A thin Layer Chromatography study of the methanol extract of Soyabean seed and Pumpkin seed using solvents like toluene, acetone, and formic acid in the ratio 4.5:4.5:1 for separation of the metabolites resulted in 2 spots excluding the standard. The Rf values are as follows: standard Rf - 0.78, spot 1 Rf - 0.76, and spot 2 Rf - 0.76.

Table 3. Phytochemical screening of methanolic extract of Soyabean seed (*G. max*) and Pumpkin seed (*C. pepo*).

Chemical Constituent	Methanolic Soyabean Seed Extract	Methanolic Pumpkin Seed Extract
Alkaloids	+	+
Glycoside	+	+
Terpenoids	+	+
Reducing sugar	+	-
Saponins	-	+
Tannins	+	+
Flavonoids	+	+
Steroids	+	-

Discussion

Microbial infections pose a serious health problem around the world. Plants and their different parts have always been an unfathomable source of therapeutic agents. World Health Organization (WHO) recommends medicinal plants as the best source of diverse drugs and active compounds [10]. Researchers need to explore their properties and understand their safety and efficiency. Our study intended to focus on the antimicrobial effect of methanolic extract soyabean (*G. max*) and pumpkin seeds (*C. pepo*) against the study microbes prevalent in the oral cavity. Both the extracts, soyabean and pumpkin seed, showed the highest zone of inhibition for *Streptococcus mutans* at 100 mg/ml, 26±0.31 mm, and 24±0.82 mm, respectively. Both the seeds were effective against *S. pyogenes* at all concentrations. Contrary to our results, Soni and Bali, in their study, found that the methanolic extract of Pumpkin seed has high activity against *B. subtillus* and *S. auereus* species [11]. In the Chaleshtori et al. [12] study, the highest diameter of the zone of growth inhibition for *S. aureus* was 19.33±1.56 mm against the methanolic extract of soyabean seed at a concentration of 100 mg/ml, for we found it to be 21±0.36 mm.

The presence of phenols, saponins, micronutrients, flavonoids, and polysaccharides in soyabean and pumpkin seeds attributes to their antimicrobial activity. Both extracts do not possess antifungal activity, and this result agrees with Soni and Bali's study [11]. Ghahari et al. [13] have tested the antifungal activity of soyabean seed against *Pyricularia oryzae*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, and found it to be effective.

The present study showed that the soyabean seed extract has an antimicrobial action with MIC values fluctuating from 1.56-4.5 mg/ml against dental pathogens, and pumpkin seed extracts showed antimicrobial action with MIC values fluctuating from 3.5-5.0 mg/ml against dental pathogens (Table 2). The MBC values were recorded as 11.5-15.0mg/ml for soyabean seed extracts and 12.5-17.0 mg/ml for pumpkin seed extract. The MIC values for soyabean seed methanolic extract against *S. mutans* (1.56 mg/ml), *S. pyogenes* (3.0 mg/ml), *S. aureus* (3.5 mg/ml), *Lactobacillus* (4.5 mg/ml) and *Candida albicans* (1.56 mg/ml).

In their study, Ghahari et al. [13] found that the essential oil of soyabean seeds showed maximum activity against *R. toxicus* and *P. syringae* subsp. *syringae* with MIC=25 µg/mL, followed by *S. aureus* (MIC = 50 µg/mL), *B. subtilis* and *E. coli* (MIC = 100 µg/ mL), *P. aeruginosa*, *P. viridiflava*, and *X. campestris* pv. Campestris (MIC=No inhibition with the highest concentration in the test conditions). They stated that the alkaloid extracts of soyabean seed have antimalarial, antimicrobial, anti-hyperglycaemic, anti-inflammatory, and pharmacological effects. They are good spasmolytic and anaesthetic agents [13].

Researchers revealed that the methanolic extract of Pumpkin seed showed a maximum presence of terpenoids, phenolic compounds, tannins, and a small amount of Coumarins, which have been reported to have multiple biological effects, including antioxidant activity [5,6]. Alves et al. [14] and Saavedra et al. [15] showed that syringic, coumaric, ferulic, and vanillic acids, which were found in the soyabean seed extract, exhibited significant antimicrobial activity against both Gram-positive and Gram-negative bacteria. In contrast, a few studies have also revealed that the methanolic extract of soyabean seed is more effective on Gram-positive bacteria than Gram-negative bacteria [11].

Kumar et al. [16] reported that zone of the growth inhibition of crude and pure extract of soyabean seed extract against *B. subtilis*, *E. coli*, *Vibrion harvey* (*V. harvey*), *Aeromonase hydrophila* (*A. hydrophila*) and *V. patahaemolyticus* were 14.07±1.20 and 15.54±0.5 mm, 9.05±0.5 and 13.17±0.2 mm, 8.02±0.6 and 16.07±0.8 mm, 9.47±0.2 and 14.32±0.2 mm, and 10.84±0.6 and 15.49±0.7 mm, respectively. They showed that the high presence of saponin is the main factor for the high antimicrobial activities of soyabean seed. They also reported that the zone of the growth inhibition of 1 g/mL, 2 g/mL, and 3 g/mL concentrations of the soyabean seed against *E. coli*, *P. aeruginosa*, and *V. Harvey* was 4.2, 5.9, and 2.7 mm, 3.8, 5.4 and 1.4 mm and 5.4, 6.1 and 1.8 mm, respectively.

In the present study, qualitative phytochemical analysis of the methanolic soyabean seed extract revealed the presence of alkaloids, glycosides, terpenoids, flavonoids, reducing sugars, tannins, and steroids were confirmed, but saponins were absent, while the pumpkin seed extract as the presence of alkaloids, glycosides, terpenoids, flavonoids, tannins, and saponins was confirmed, but reducing sugars and steroids were absent. The biological impact of soyabean seed has been attributed to the phenolic compounds present. Flavonoids in soyabean seeds show a wide range of biological activities, such as inhibition of cell proliferation, induction of apoptosis, inhibition of enzymes, and antibacterial and antioxidant activities, and their remarkable effects on human nutrition and health [16]. The mechanism of action of flavonoids may be through scavenging or chelating processes.

Conclusion

Considerable antimicrobial effects of the methanolic extracts of soyabean seed and pumpkin seed against pathogenic bacteria have been identified, especially *S. mutans* and *S. pyogenes*. Both seed extracts have vital chemical constituents that have an antimicrobial effect.

Authors' Contributions

RN	https://orcid.org/0000-0002-0253-7720	Conceptualization, Methodology, Formal Analysis, Writing - Original Draft and Writing - Review and Editing.
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MJ	https://orcid.org/0000-0003-1189-3214	Conceptualization, Methodology, Formal Analysis and Writing - Original Draft.
AM	https://orcid.org/0000-0001-6970-600X	Conceptualization, Methodology, Data Curation and Writing - Review and Editing.
All authors declare that they contributed to a critical review of intellectual content and approval of the final version to be published.		

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None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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