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Identification of oral bacterial species from Buccal cavity of Oral Cancer patients compare to normal individuals, and antibacterial efficacy testing of 'Soursop' leaves extract and its Silver and Chitosan based nanoparticles (CSNPs) against these oral bacteria".

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ABSTRACT:

Background: The aim of this study was to describe the role of Oral bacteria in the severity of Oral Cancers as compared to normal individuals. Many pathogens that can have an impact on cancer development in the gastrointestinal tract are also found in the oral cavity. Some specific species have been identified that correlate strongly with oral cancer, and to screen the antibacterial efficacy of **'Soursop'** leaves extract against these oral bacterial species and we identified antioxidant activity of these extracts.

Methods: Study started with health history survey of selected individuals having Oral Cancer & Healthy individuals. A mixed group of male & female with age group of 18 to 75 years from Bhopal and its peripheral regions, (M.P) India, after final approval received by 'Institutional Ethical Committee' (Approval. No: 1130e/JNCH/RES/20/12/24).

Screening of individuals: With the help of Department of Surgery, JNCH & RC, Bhopal, we identified the Oral cancer patients and after taking their informed consent our nursing staff took the oral sample with all aseptic precautions for microbial culture, healthy individuals were randomly chosen with few questions about their habits and disease history. Oral swab (Sterile) was used to collect the samples for further experiments.

Experiments performed: Bacterial culture of swab samples taken from enrolled subjects on different agar medium, Morphological observation of bacterial colonies, Biochemical identification of isolated bacteria, Gram staining for microscopic identification, Disc & Agar well diffusion method for anti-microbial susceptibility testing of 'Soursop' leaves extract against these oral bacterial species, and antioxidant activity were calculate by DPPH method.

Results: The sample size consisted of 25 Oral Cancer patients and 10 healthy controls, including both males and females. The present study yield a diverse array of bacterial species from the collected samples confirmed after gram staining and biochemical test. Aqua- Alcoholic extract of *Annona muricata* L. by using DDW and 95% Methanol was tested against these bacteria, another preparation subjected to nanoparticles/ metallic conjugate by using (Silver nitrate & Chitosan polymer) among the selected solvents and prepared metallic conjugates and nanoparticles all of these exhibited significant (mild to moderate) antibacterial and antioxidant effect.

Conclusion: In this study, we aimed to determine the relationships between bacterial profile of buccal cavity among oral cancer subjects and healthy individuals. Through the analysis, we found significant changes in bacterial species between tumour sites and normal tissues in the buccal mucosa, and good antibacterial effects of 'Soursop' leaves extract observed against these oral bacterial species.

Key words: Buccal cavity, Oral Cancer, antibacterial effects, *Annona muricata* L.

INTRODUCTION:

There is a strong association between oral diseases and microbial involvement. The environment of oral cavity is an open, dynamic systems with diverse conditions of specific specialized surfaces of soft tissue as well as hard structures. As the natural products those derived from plants have been used to help humankind maintain its health. Over the last century, plant phytochemicals are an important key for the pharmaceutical, dentistry microbial cancerous discovery. *A. muricata* L. extract-mediated green synthesis proposed in this work is a fast, economical and effective technique to synthesize NPs with promising antibacterial activity a well published work done by (**Vidhya. S, et al., 2022**). As per reviews several studies have proven the plant *A. muricata* (Soursop) is having anti-bacterial & antioxidant properties.

MATERIAL & METHODS:

After IEC approval of this study collected samples were subjected for bacterial culture on different agar medium after 24 hrs incubation morphological observation of bacterial colonies was performed, and isolated colonies were picked for biochemical identification of isolated bacterial, Gram staining for microscopic identification, Disc & Agar well diffusion method for anti-microbial sensitivity testing of 'Soursop' leaves extract against these oral bacterial species. This plant is widely found in Southeast Asia, South America, and African rainforests. *A. muricata* belongs to the family Annonaceae. (**N. Bhattacharjee, et al., 2022**) in addition to get more information we performed antioxidant analysis by DPPH method to analyse free radical scavenging activity of above extracts and its nanoparticles.

Collection of Plant sample:

The Soursop leaves (*Annona muricata*) was collected from the trees planted in Madan Mohan Herbal Garden of JNCH & RC, Bhopal in the month of February'2025. After collection and washing of the leaves, they were separated from the twigs and dried in shade for further preparation.

Preparation of extracts:

Aqueous & Methanolic extracts of the Soursop leaf were prepared in the Pharmacology lab, Department of Research & Clinical Genetics, JNCH & RC, Bhopal, India. Maceration method is used for extraction as it is a convenient, simple inexpensive, and favourable technique, especially in the case of small-scale extraction, such as that at laboratory scale. The alcoholic extract had the good antioxidant activity compared to the fresh decoction and juice study conducted by. (**Eva Fransiska *et al*., 2023**). Antioxidant analysis by using DPPH method a modified method adopted from (**Mensor et al., 2001**) and (**Manigauha et al., 2009**).

Silver Conjugation Synthesis:

- Synthesis of Silver Nanoparticles with extracts of *A. muricata,* a modified method adopted from (**Asif M. et al., 2022**).

Chitosan based NPs preparation:

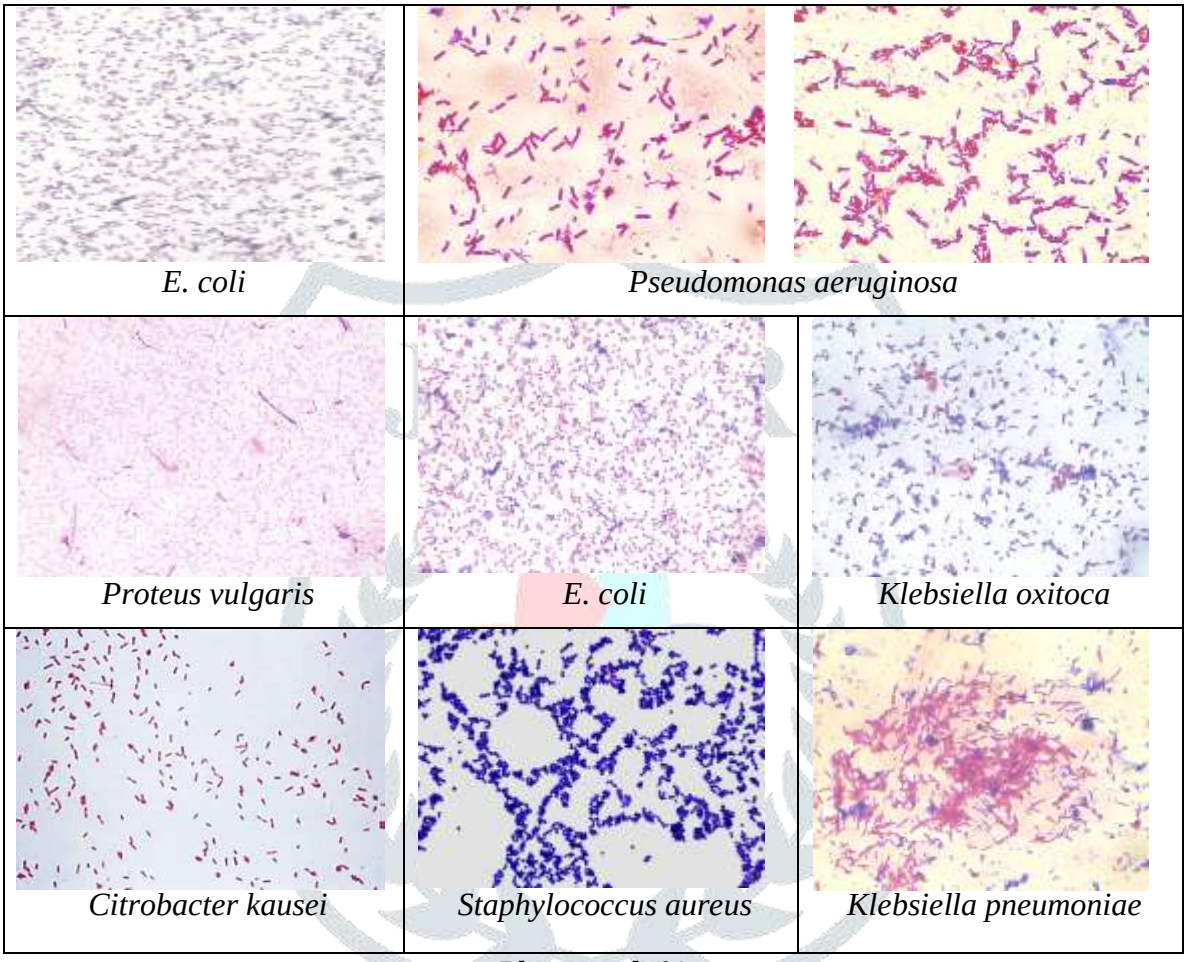
- A modified method adopted from (**B. Ozdamar. et al., 2023**).

Methods for Antimicrobial susceptibility testing:

- Disc diffusion or 'Kirby-Bauer' method
- Agar well diffusion method.

Microscopic analysis: Motic BA-210 microscope with imagining system with 40 to 100 X magnification was used.

OBSERVATIONS:



Photograph:01

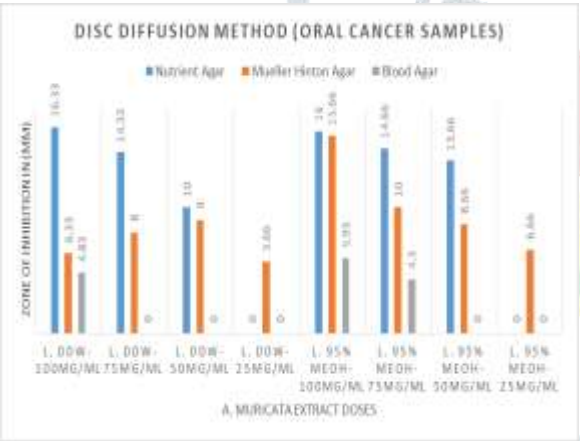
Showing images of different bacterial species isolated from Oral swab method of Oral cancer patients. Gram stain images taken by MoticBA-210 image analyser 40X -100X magnification.

Table: 01 Showing the average colony frequency of the bacterial species in oral samples of '**Normal individuals**' after confirmation by biochemical test and Gram staining results:

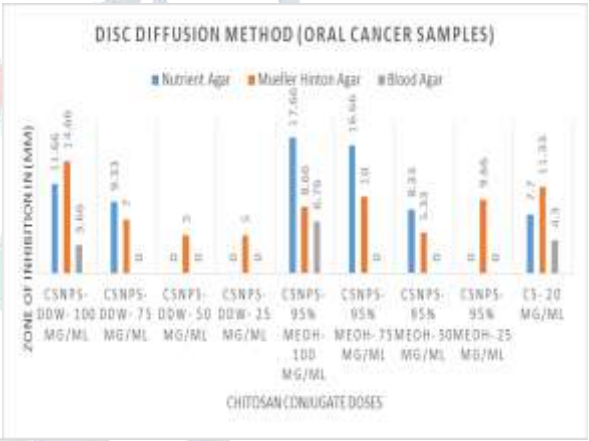
S. No.	Isolate from (Normal)	Gram Staining	Percentage %
1	*Citrobacter kausei*	-	4
2	*E. coli*	-	34.1
3	*Klebsiella oxitoca*	-	8
4	*Klebsiella pneumoniae*	-	23.5
5	*Morgenlla morganii*	-	1
6	*Proteus vulgaris*	-	9
7	*Pseudomonas aeruginosa*	-	20.4

Table: 02 Showing the average colony frequency of the bacterial species oral samples of **'Oral cancer patients'** after confirmation by biochemical test and Gram staining results:

S.No	Isolate from (Oral Cancer)	Gram staining	Percentage (%)
1	*Robacter kausei*	+	3.7
2	*Prevotella intermedia*	-	5
3	*Porphyromonas gingivalis*	-	2
4	*Escherichia coli*	-	26
5	*Klebsiella oxytoca*	-	7.1
6	*Klebsiella pneumoniae*	-	12.5
7	*Staphylococcus aureus*	+	8.9
8	*Pseudomonas aeruginosa*	-	34.8

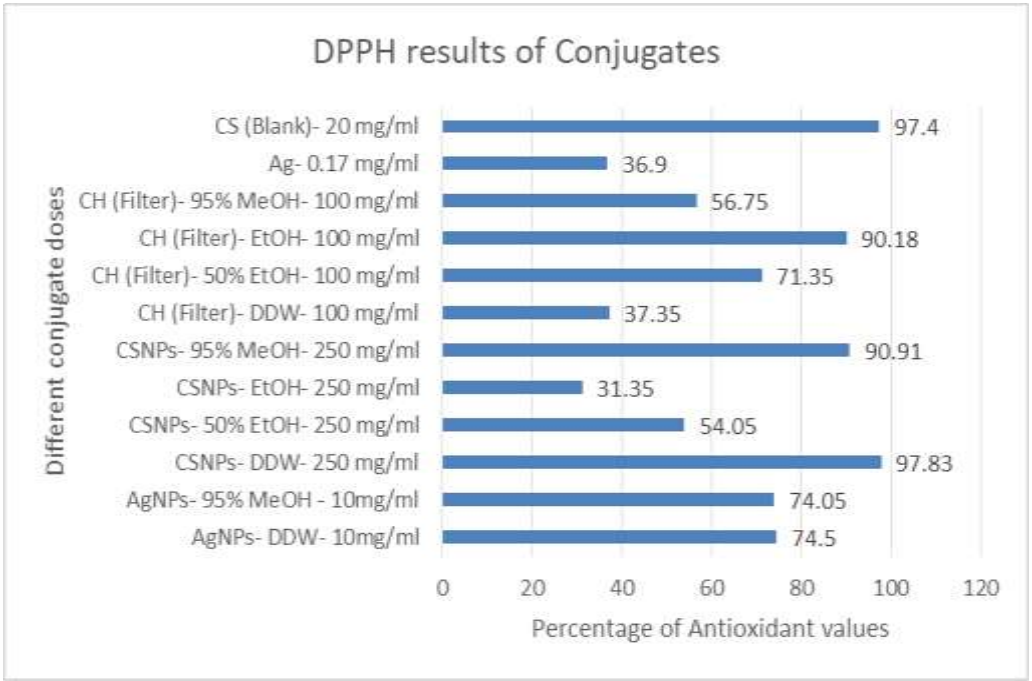


Graph: 01



Graph: 02

Showing zone of inhibition (in mm) by disc diffusion method of oral cancer patients (*Annona muricata* extract doses, Chitosan nanoparticles doses)



Graph: 03 Showing percentage of Antioxidant values of different conjugates by DPPH methods.

RESULT:

The present study yield a diverse array of bacterial species from the collected samples. These cultures served as the foundation for further comprehensive investigations and analyses. Hence, after the successful isolation of pure cultures of bacterial species, individual colonies were meticulously studied for morphological, microscopic and biochemical characteristics. The oral bacteria of 'Healthy individuals' was analysed and the frequency of bacterial species in swab samples is shown in (**Table: 01)** the most prevalent bacterial species was *E. coli*, which was (34.1%) after taking average of counted colonies. The second most common species was *Klebsiella pneumonia* (23.5%). *Pseudomonas aeruginosa* was the third most frequent species with (20.4%). Other bacterial species, such as *Citrobacter kausei, Klebsiella oxytoca, Morganella morganii,* and *Proteus vulgaris*, were less abundant and detected less than 10%. The results of this study revealed that the salivary microbiota (bacterial) of healthy individual were dominated by a few bacterial species, mainly *E. coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*.

Bacterial species prevalence in Oral cancer patients:

The (**Table:** 02) showing the frequency of the bacterial species isolated from swab samples of Oral cancer patients. The most common species was *Pseudomonas aeruginosa*, with average colony count (34.8%), *Escherichia coli* was the second most common species with (26%). *Klebsiella pneumoniae* (12.5%), followed by *Staphylococcus aureus* (8.9%) and *Klebsiella oxytoca* is (7.1%) etc. All of these results are well checked by biochemical testing & Gram staining. Microscopic images after Gram staining shown in (**Photograph:01**).

Comparison of bacterial isolates between Normal and Oral cancer:

The results suggested that the oral microbiota of Oral cancer patients was characterized by a high abundance of *Pseudomonas aeruginosa* some previous studies have reported that *Pseudomonas aeruginosa* is a common pathogen in the respiratory tract and can cause chronic infections, *E. coli* and *Klebsiella pneumonia* were common in both groups, these bacteria are known to be opportunistic pathogens that can cause infections in immune-compromised hosts or in damaged tissues. The results also showed that some bacterial species, such as *Citrobacter kausei, Klebsiella oxytoca,* and *Morganella morganii,* were detected in low frequency. These bacteria are also considered as potential pathogens that can cause infections in various sites of the human body.

Anti-bacterial activity of leave extract of '*Annona muricata* L' against oral bacteria:

Aqua- Alcoholic extract of *Annona muricata* L. by using DDW and 95% Methanol was tested against bacteria collected by oral swab culture, another preparation with these extracts are subjected to nanoparticles/ metallic conjugate by using (Silver nitrate & Chitosan polymer) among the selected solvents and prepared metallic conjugates and all of these test were repeated three or more times to get their mean values and prepared nanoparticles exhibited significant (mild to moderate) antibacterial effect. However, 95% MeOH extract

exhibited **16.0 ± 7.39** at Nutrient agar. Values obtain **15.66 ± 3.13** at Muller Hilton agar (Mean ± SE) and 5.93 ± 0.466 by using Blood agar medium all values are in (mm) zone of inhibition against bacterial growth after oral swab culture (Graph:01).

Higher concentration of these extracts shows excellent inhibitory activity as compared to the standard control (Antibiotics) like AMP-10 and PIT 100/10 and the prepared conjugates CSNPs- 95% MeOH- 100 mg/ml shows good 17.66 ± 6.59 mm, zone of inhibition at nutrient agar AgNPs- 95% MeOH- 100 mg/ml also shows moderate effect with more than 10 mm zone of inhibition graphically represent in (Graph: 02). Charcoal filter (CH) extract shows very mild effect against these bacteria.

Our results about DPPH based antioxidant test after three repetition and comparison with Standard control (Ascorbic Acid 0.025mg/ml) and different solvents used in the sample preparation with calculated (Mean ± SD) values of inhibition and percentage antioxidant activity with P-value: 0.023 at significant level (<0.05) the graphical presentation of antioxidant percentage shown in (Graph:03) with absorbance of 0.189 ±0.0078 (Mean ± SD) at 517nm of Standard control (Ascorbic Acid 0.025mg/ml) shows 56.70% antioxidant activity near to IC_{50} value and the test groups like DDW extract (100mg/ml), 95% MeOH (100mg/ml) gives above 80% antioxidant activity alone Ag very minutely neutralize free radicals but with conjugates it shows increase activity of more than 70%. Chitosan polymer shows good antioxidant activity alone 0.0136 ±0.00237 (73.4%) the Chitosan based nanoparticles with DDW extract shows excellent antioxidant activity 97.83% on 100mg/ml concentration.

CONCLUSION & DISCUSSION:

In this study, we aimed to determine the relationships between oral buccal mucosal microbial profile of oral cancer subjects and normal individuals. Through the analysis, we found significant changes in bacterial species between tumour sites and normal tissues in the buccal mucosa.

Bacteria of the oral cavity play an important role in the development of oral, colorectal, and pancreatic cancers. The most well confirmed is the carcinogenic effect of oral periopathogens: *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. Others playing an essential role in cancerogenesis seem to be *Streptococcus sp*., *Peptostreptococcus sp*., *Prevotella sp*., and *Capnocytophaga gingivalis*. Bacteria can have an oncogenic effect on human cells in three ways: leading to chronic inflammation, acting as an antiapoptotic, and producing carcinogenic substances. However, further research is needed to clearly define specific oral bacteria as carcinogens. The oral microbiota plays an important role in the human microbiome and human health, and imbalances between microbes and their hosts can lead to oral and systemic diseases and chronic inflammation, which is usually caused by bacteria and contributes to cancer.

As per our study data, we compared the Buccal cavity bacterial profiles of the Oral cancer subjects and Normal control groups there is significant differences in microbiota between the two groups shown in (Table:1&2). As a part of the digestive tract, oral cavity includes diverse microorganisms (Segata et al., 2012), and oral microbiota is a complex microbial community (Lamont et al., 2018). The oral microbiota plays an important role in human

health, and dysbiosis of oral microbiota can lead to a variety of systemic diseases (Olsen and Yamazaki, 2019). Since changes in gut microbial composition may contribute to cancer initiation and progression (Vivarelli et al., 2019), oral microbial dysbiosis may also be involved in the occurrence and development of oral cancer.

All higher concentrations of *Annona muricata* leave extracts were effective on the microbiota except bacteria grown on Blood agar medium. The Soursop extract was highly effective on these species, these concentrations exhibiting bactericidal property with conjugates & prepared NPs and the effect was statistically significant ($p<0.05$). Data obtained was analysed using one way analysis of variance (ANOVA). The antimicrobial activity of plant extracts is majorly linked to the presence of phytochemicals. These anti-bacterial inhibitions give credence to the fact that *Annona muricata* leaf extract exhibit antibacterial activities.

FUTURE PROSPECTIVE:

Soursop leaf extract has potent antibacterial activities against pathogenic oral microbes. *In vitro* testing of soursop leaf extract shows good results with its efficacy depending on the concentration used. Further studies are recommended to validate this result and establish the extent to which soursop extract can be used in decreasing the pathogenicity of oral tumours. Finally, to answer the original question paused in the title, after reviewing all these studies, we can with much certainty affirm that it is true, the natural therapy to most disease conditions including cancer is growing in our back yard, especially in the Tropics. It only requires big steps to be taken for more detailed studies and advocating for pharmaceutical development or local formulations that may not necessarily be a replacement of the current treatment regimens, but where the extracts of *A. muricata* have shown better and promising activity like in cancer and other non-communicable diseases, be used to help save the human race.

Despite the extensive experiences in use of medicinal plants in traditional medicine, scientific study and identification of active plant compounds and their effects can lead to the discovery of new therapeutic benefits and the production of nature-based products in the future. To achieve this purpose, extensive research especially in conjugate and nanoparticle based products is fundamentally important to control the quality of raw drugs and the formulation to justify their use in the modern medicine system; subsequently, animal studies and clinical trials are required to use the benefits of these plants. In addition, in the development of medicine from medicinal plants, among other things, a practical is required by using the natural compounds in their proved form, rather than taking years trying to develop a patentable synthetic analogue as the human race suffers, may be the best service we might ever give to this world.

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Statements & Declarations:

With proper Ethical approval all procedures performed in the study involving human participants were conducted according to the ethical standards of the institutional ethics committee as per guidelines of the 1975 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved with approval No: (1130e/JNCH/RES/20/12/24). Informed consent before data collection, written informed consent was obtained from all subjects participating in the study. To comply with ethical considerations, all medical record information was kept confidential. The names and surnames of participants were not used for data collection, and data collection was carried out after obtaining the ethical approval certificate code of ethics from IEC, Jawaharlal Nehru Cancer Hospital, Idgah Hills, Bhopal, (M.P) India.

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Competing Interests:

"The authors have no relevant financial or non-financial interests to disclose."

Author Contributions:

"Authors [**Pulkit Raikwar & Sarfaraz Hanfi**] contributed to the study experimental part. Material preparation, data collection and analysis were performed by [**Sarfaraz Hanfi & Alibha Guru Rawat**]. [**Renu Singh**] permitted to arrange samples from surgical ward, JNCH & RC. The first draft of the manuscript was written by [**Sarfaraz Hanfi & Pulkit Raikwar**] checked by [**Ankur Chhari, Shivani Tomar**]. [**Pratima Sharma**] is dissertation mentor form IEHE, all authors commented on previous versions of the manuscript all authors read and approved the final manuscript."

Ethics approval:

"This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Jawaharlal Nehru Cancer Hospital, Idgah Hills, Bhopal, (M.P) India. (Date 20/12/2024, Approval No. 1130e/JNCH/RES/20/12/24)."

Consent to participate:

"Informed consent was obtained from all individual participants only their Health history & oral swab sample was used in the study."

Consent to publish:

No photographs and images of any participants are added in the submitted manuscript.

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