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CHARACTERIZATION OF FRUIT EXTRACT OF *Terminalia chebula* AND ITS ANTIBACTERIAL ACTIVITY AGAINST *Porphyromonas gingivalis* ISOLATED FROM PERIODONTITIS

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ABSTRACT

Terminalia chebula is one of the medicinal plants studied extensively for their antimicrobial properties. *Porphyromonas gingivalis* is one of the common bacterial etiologies for periodontitis. The study aimed to characterize the fruit extract of *Terminalia chebula* and test its antibacterial activity against *Porphyromonas gingivalis.* Of the 20 periodontitis samples processed, 13 were positive for *P. gingivalis.* The ethyl acetate extract showed better anti-*P. gingivalis* activity and a greater number of phytochemicals compared to methanol and water. 1,2,3 Benzenetriol was a major compound present in both ethyl acetate and methanol extracts.

Keywords: Periodontitis, *T. chebula, Porphyromonas gingivalis,* FTIR, GCMS.

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INTRODUCTION

Periodontitis is a persistent inflammatory disease affecting the supportive structures of the tooth, characterized by infectious elements. Its incidence is more intense in smokers, who experience increased alveolar bone loss, tooth loss, attachment loss, and tooth mobility. Periodontal infection results either from the penetration of pathogenic microbes in the tissue or by activating already existing germs that are not pathogenic under normal conditions. Aggressive periodontitis is multifactorial and complex, with viral and bacterial etiology, host immune response, and genetic components playing a role. The two common bacteria encountered in periodontitis in younger populations are *Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans*.[1] *P. gingivalis* is commonly found in the human body, especially in the oral cavity. *Porphyromonas sp.* is a gram-negative, non–spore–producing anaerobic bacilli, which produces porphyrin pigments (dark brown/black pigments) on blood agar. Chronic infections associated with periodontitis, act as a predisposing factor for premature birth, underweight newborns, heart disease,[2] diabetes,[3] obesity,[4] rheumatoid arthritis,[5] and metabolic syndrome.[6] *P. gingivalis* can be easily translocated to other tissues when fed orally, and it was detected in the brains of Alzheimer patients.[7,8] Mechanical therapy alone cannot eliminate *P. gingivalis* from the pockets of periodontal tissue. Antimicrobial treatment will reduce the periopathogen and enhance the benefits accrued due to conventional mechanical treatments.[9] But, due to the development of drug resistance, systemic management of antimicrobials is ineffective. Plants with medicinal properties serve as a source of therapeutic agents. Numerous plant products have been explored for their antimicrobial activity against many pathogenic bacteria in the oral cavity.[10,11] One such plant is *Terminalia chebula*. It is used to treat various ailments and diseases, but research on its activity against *P. gingivalis* is limited. Hence, the present investigation aims to characterize the fruit extract of *T. chebula* and evaluate its antibacterial activity against *P. gingivalis.*

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EXPERIMENTAL

Sample Collection

Periodontal pocket fluid was collected from patients visiting the periodontics department of Sathyabama Dental College & Hospital with periodontitis with the assistance of a periodontist by using a sterile absorbent paper point. Paper strips were placed at a depth of 4 mm in the pocket for 60 seconds until mild resistance was felt. [12] It was placed in a Brucella selective supplement (HiMedia) transport medium and brought to the laboratory immediately. Informed consent was obtained from the patient for sample collection. Institutional human ethical clearance was obtained to collect patient specimens (Sathyabama University/IHEC/Study No. 029, dated 2018). Inclusion criteria for sample collection were patients with known clinical symptoms of periodontitis, and exclusion criteria were patients on antimicrobials for a week before sample collection and patients on fluoride treatment for two weeks before sample collection. Twenty samples were collected for the isolation and identification of *P. gingivalis* over three months.

Specimen Processing and Identification of *P. gingivalis*

The periodontal fluid of 100 µl was plated on Brucella blood agar (HiMedia) by confluent streaking and incubated for 72 hours in an anaerobic jar using a gas pack.[13] *P. gingivalis* was identified based on the morphology of colonies obtained on Brucella blood agar, gram stain morphology, and biochemical tests such as growth in ox bile, indole test, catalase test, urease test, and nitrate reductase test, according to the protocol described in Bailey and Scott's Diagnostic Microbiology under anaerobic conditions and susceptibility to antibiotics: colistin (10 µg), kanamycin (1000 µg), and vancomycin (5 µg).[14]

Preparation of Fruit Extracts

T. chebula fruit procured from the herbal shop was washed, air-dried, and ground to a fine powder. About 30 gm of powder was used for extraction with methanol, ethyl acetate, and water as solvents, with the help of a Soxhlet apparatus. A rotary drum evaporator condensed the extract, and it was dried in a desiccator.[15]

Antibacterial Study of Extracts against *P. gingivalis* by Well Diffusion Method

The suspension of *P. gingivalis* was made in physiological saline. A sterile swab was dipped in culture suspension and used to obtain lawn cultures on Brucella blood agar. Wells were punched on Brucella blood agar with a sterile cork borer. Undiluted extract of volume 100 µl was loaded into the well, and plates were placed in an anaerobic jar for incubation at 37^0C for 3 to 5 days. The extract's activity was assessed by reading the inhibition zones around the wells.[16] The extracts showing a zone of inhibition above 10 mm were subjected to the minimum inhibitory concentration (MIC).

Determination of MIC

Extracts were diluted using appropriate solvents from 1/2 to 1/256. The percentage of extract in each dilution is listed in Table-1. An extract volume of 100 µL from each dilution was loaded in wells punched on Brucella blood agar with a lawn culture of *P. gingivalis.* Plates were placed in an anaerobic jar for a period of 3 to 5 days at a 37^0C incubator, and the zone of inhibition obtained was recorded. The MIC was determined as the reciprocal of the highest dilution of extract that displayed a zone of inhibition. Five strains were subjected to MIC. This method was used to estimate the least amount of extract required to inhibit the growth of bacteria.[16]

Phytochemical Analysis of Aqueous Extract

Secondary metabolites present in the extracts were detected using the standard procedure mentioned by Harborne *et al*.[17]

Determination of Total Phenols, Flavonoids, Alkaloids, Saponins, and Terpenoids

Folin-Ciocalteu method for phenol, a colorimetric method using aluminum chloride for flavonoids, the procedure by Sreevidya N *et al*., for alkaloids, the method described by Kareru *et al.,* for saponins, the procedure described by Ghorai *et al.,* for terpenoids were used.[18-22]

Detection of Bioactive Compounds

It was done by GCMS and FTIR. They are high-resolution analytical tools used to identify functional groups and elucidate structural compounds. They were performed using standard protocols.[23,24]

RESULTS AND DISCUSSION

P. gingivalis Isolation and Identification

Among the 20 periodontal pocket fluid specimens processed, 13 were positive for *P. gingivalis* (13/20, 65%), which were identified based on black pigmented colonies on Brucella blood agar (Fig.-1A), gram stain showing gram-negative bacilli that are non-sporing, absence of fluorescence when exposed to UV light, resistance to colistin, kanamycin, susceptibility to vancomycin, no growth in ox bile, positive for the indole test and negative for the catalase test, nitrate reductase test, and urease test. Griffin *et al*., isolated *P. gingivalis* in 79% (103 of 130) of the periodontitis group, whereas it was detected only in 25% (46 of 181) of the healthy subjects. [25] A prevalence rate of 61.96% was reported by Rodriques *et al*.[26]

Activity of *T. chebula* Fruit Extract Against *P. gingivalis*

Water, ethyl acetate, and methanol were used as solvents to extract active ingredients from the fruit of *T. chebula.* The maximum zone size obtained by the well diffusion method for the three extracts was mentioned in Fig.-1: B, C, D. For the 10 strains tested, the zone size for ethyl acetate extract varied from 40 to 45 mm, for methanol extract, the zone size varied from 33 to 40 mm and aqueous extract produced zones that varied from 12 to 17 mm. Among the three solvents used in the study, ethyl acetate extract exhibited a bigger zone of inhibition of 45 mm, followed by methanol extract (40 mm), whereas the aqueous extract showed the smallest zone size of 17 mm by the well diffusion method. When three extracts were subjected to MIC, ethyl acetate extract showed a MIC of 3.12 %, followed by methanol extract (6.25%), and aqueous extract showed activity only in undiluted concentrations (MIC value of 100%) (Table-1, Fig.-2,3). All five strains tested showed the same MIC values for all three extracts. This concluded that ethyl acetate extract possessed better activity against *P. gingivalis*. Nevertheless, a study done by Baliah *et al*., on bacteria other than *P. gingivalis* showed methanol, along with ethanol and acetone, as the best among the 11 solvents used.[27]

Table-1: MIC Results of Three Solvent Extracts of *T. chebula* Against *P. gingivalis*

S.No.	Dilution of the extract	Percentage of extract in dilution	Inhibition zone in millimeters (mm)		
			Aqueous extract	Ethyl acetate extract	Methanol extract
1	Neat	100%	17	44	40
2	1/2	50%	-	30	30
3	1/4	25%	-	25	28
4	1/8	12.5%	-	24	25
5	1/16	6.25%	-	23	16
6	1/32	3.12%	-	20	-
7	1/64	1.56%	-	-	-
8	1/128	0.78%	-	-	-
9	1/256	0.39%	-	-	-
10	1/512	0.19%	-	-	-

(-) - no zone obtained

According to *Parek* and Chanda, the maximum zone of inhibition was obtained for ethanol extract, while the minimum zone size was recorded for petroleum ether extract of *T. chebula,* when tested against standard strains of bacteria.[28] Dental plaque bacteria were considerably reduced by ethanol extract.[29] The efficacy of *T. chebula* organic and aqueous fruit extracts was observed against *Staphylococcus aureus*, *Pseudomonas aeruginosa,* Acinetobacter *sp, Escherichia coli,* and *Proteus mirabilis*.[30] Methicillin-resistant *Staphylococcus aureus* (MRSA), multiple drug resistant *P. aeruginosa* and *Acinetobacter* sp., beta-lactamase producing-*E. coli* were killed by aqueous and methanol extracts but were not active against multi-drug resistant *Klebsiella pneumoniae*.[31] To the best of our ability, the activity of *T. chebula* fruit extract on *P. gingivalis* has not been reported by other authors.

Phytochemical Analysis of the Fruit Extract of *T. chebula*

The ethyl acetate extract contained the most elements, with seven, followed by six in methanol and five in aqueous extract (Table-2). Glycosides were absent in all three extracts screened. Apart from this,

carbohydrates were absent in ethyl acetate extract, carbohydrates, and terpenoids were absent in methanol extract, and steroids, saponin, and terpenoids were absent in aqueous extract.

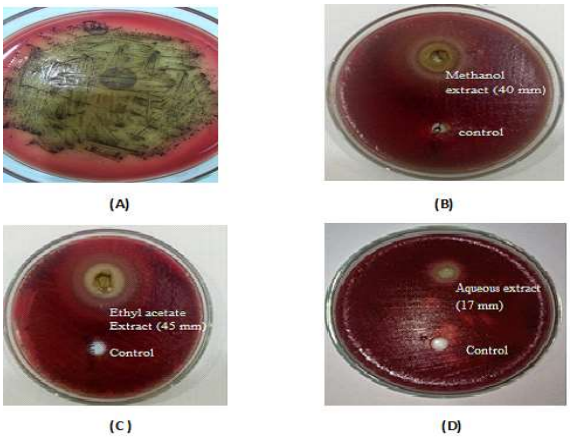

Fig.-1: (A) Black Colonies of *P.gingivalis* on Brucella Blood Agar, (B) - Well Diffusion for Methanol Extract Against *P.gingivalis,* (C) - Well Diffusion For Ethyl Acetate Extract Against *P.gingivalis,* (D) Well Diffusion for Aqueous Extract Against *P.gingivalis*

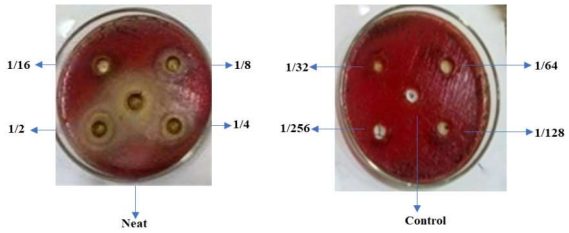

Fig.-2: MIC Results of Methanol Extract

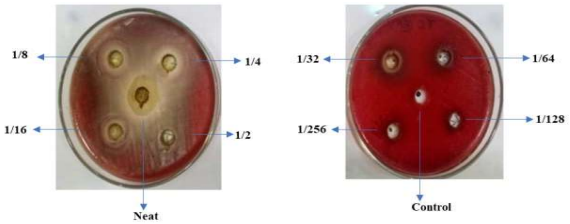

Fig.-3: MIC Results of Ethyl Acetate Extract

Vemuri *et al*. demonstrated the absence of glycosides and carbohydrates from methanol extract, similar to our study, but they also showed the existence of terpenoids and the absence of saponins, which is reversed in our study. Likewise, for ethyl acetate extract, carbohydrates and glycosides were absent, similar to our study, but they also reported the absence of saponins and alkaloids. Steroids and saponins were absent in the aqueous extract of our study, whereas they were present in their study.[32] There was a difference in the phytochemical analysis of all three extracts reported by Baliah *et al*.[27] Tarik *et al*. reported the existence of alkaloids, flavonoids, tannins, carbohydrates, proteins, phenols, and terpenoids in methanol extract. In contrast, our study found the absence of carbohydrates and terpenoids.[33]

Total Estimation of Phytochemicals

In ethyl acetate extract, terpenoids were seen in greater quantity followed by flavonoids, alkaloids, polyphenol, and saponin. Methanol extract showed more polyphenols, followed by flavonoids (Table-3). Genwali *et al.* demonstrated a greater amount of phenolic content in the methanol extract compared to the acetone extract of *T. chebula*.[34] Shaha and Verma demonstrated total phenol (134.47 mg), flavonoids (7.934 mg), and tannin (31.47 mg) from polyphenol extract.[35]

Table-2: Phytochemicals Identified from the Fruit Extract of *T. chebula*

S. No.	Name of the phytochemical	Tests	Aqueous extract	Ethyl acetate extract	Methanol extract
1	Alkaloids	Mayer's test	+	+	+
2	Flavonoids	Alkaline test	+	+	+
3	Steroids	Salkowski test	-	+	+
4	Saponins	Froth forming test	-	+	+
5	Phenols	Lead acetate test	+	+	+
6	Glycoside	Keller Kiliani test	-	-	-
7	Carbohydrates	Fehling test	+	-	-
8	Proteins	Ninhydrin test	+	+	+
9	Terpenoids	Horizon test	-	+	-

Present: (+), Absent:(-)

Table-3: Total Estimation of Various Phytochemicals from Ethyl Acetate and Methanol Extract

S. No.	Phytochemical	Amount (mg/100g) in ethyl acetate extract	Amount (mg/100g) in methanol extract
1	Total polyphenol as gallic acid equivalent	2.36	85.36
2	Total flavonoids as equivalent to quercetin	10.12	4.12
3	Saponins	1.12	0.89
4	Total alkaloids	3.56	0.23
5	Terpenoids	23.24	0.08

FTIR Results

Since the MIC of the aqueous extract was high (100%,) which showed activity only in undiluted extract and no activity in any of the dilutions, it was not included in the FTIR analysis. The functional groups obtained from ethyl acetate and methanol extracts are listed in Tables-4 and 5. Methanol extract showed the presence of alkynl and alkane groups, which were absent in ethyl acetate extract. Similarly, the chloride group present in the ethyl acetate extract was absent in the methanol extract. These functional groups are attributed to the medicinal properties of *T. chebula*.

Table-4: Functional Groups Obtained by FTIR for Ethyl Acetate Extract of *T. chebula*

S. No.	Peak values	Functional groups
1	3290.96 cm^{-1}	Alcohol/phenolic OH group
2	2974.72 cm^{-1}	Carboxylic acid O-H stretch
3	2888.12 cm^{-1}	Carboxylic acid O-H stretch
4	2346.63 cm^{-1}	Nitrile C≡N stretch
5	1922.01 cm^{-1}	Aromatic group
6	1382.22 cm^{-1}	C-H bending
7	1083.25 cm^{-1}	C-O ether stretch
8	1045.78 cm^{-1}	C-O ether stretch
9	880.46 cm^{-1}	C-H out of plane bending having meta substituted C-OH ring
10	665.50 cm^{-1}	C-CI group

Table-5: Functional Groups Obtained by FTIR for Methanol Extract of *T. chebula*

S. No.	Peak values	Functional groups
1	3248.63 cm^{-1}	Alkynyl C-H stretch
2	2974.83 cm^{-1}	Carboxylic acid O-H stretch
3	2885.63 cm^{-1}	Carboxylic acid O-H stretch
4	1920.11 cm^{-1}	Aromatic group
5	1380.79 cm^{-1}	Alkane C-H bending
6	1084.91 cm^{-1}	C-O ether stretch
7	1045.94 cm^{-1}	C-O ether stretch
8	880.48 cm^{-1}	C-H out of plane bending having meta substituted C-OH ring

GCMS Analysis of Fruit Extract

Since the MIC of the aqueous extract was high (100%), it was not included in the GCMS analysis. GCMS analysis of methanol and ethyl acetate extracts were listed in Table-6 and Table-7, which showed the presence of 13 and 9 compounds, respectively. Major compounds present in the methanol extract by GCMS were 1,2,3-Benzenetriol (42.52%), followed by Oleic acid (13.98%), 5-Hydroxymethylfurfural (11.23%), n-Hexadecanoic acid (4.75%). 1,2,3-Benzenetriol (36.38%) was also the major compound present in ethyl acetate extract, followed by 9,12-Octadecadienoic acid (16.49%), 9-Octadecanoic acid and Cis-Vaccenic acid (15.12%), n-Hexadecanoic acid (10.96%). 1,2,3-benzene-triol as a major compound in ethyl acetate extract was also reported by Sing and Kumar.[36] Methanol extract obtained by Subha and Diwaker reported 1,2 benzene dicarboxylic acid, mono (2 - ethylhexyl) ester as the predominant compound.[37] Further research into the toxicological evaluation of these compounds is required to develop novel chemotherapeutic agents for future use.

Table-6: GCMS Screening Results of Methanol Extract of *T. chebula*

PK#	RT	Area %	Ref	Library/ID/Database (NIST11.L)
1	7.898	0.61	11942	Naphthalene
2	8.506	11.23	11111	5-Hydroxymethylfurfural
3	9.469	0.97	23669	Thymol
4	10.567	42.52	11097	1,2,3-Benzenetriol
5	10.937	13.00	11100	1,2,3-Benzenetriol
6	11.384	5.79	11100	1,2,3-Benzenetriol
7	13.584	0.88	11047	Imidazole-4-carboxylic acid,
8	16.912	0.58	119407	Hexadecanoic acid, methyl ester
9	17.258	4.75	107549	n-Hexadecanoic acid
10	18.509	0.77	139715	11,14-Octadecadienoic acid, methyl ester
11	18.574	1.08	141291	11- Octadecadienoic acid, methyl ester
12	18.919	13.98	129338	Oleic acid
13	19.132	1.94	131262	Octadecanoic acid
14	21.748	0.75	6942	2-Piperidinone,6-methyl-beta
15	27.844	1.15	230548	Trisarsane

Table-7: GCMS Screening Results of Ethyl Acetate Extract of *T. chebula*

PK #	RT	Area %	Ref	Library/ID/Database (NIST11.L)
1	8.518	6.08	11111	5-Hydroxymethyl furfural
2	10.646	36.38	11097	1,2,3-Benzenetriol
3	10.944	3.59	11100	1,2,3-Benzenetriol
4	17.294	10.96	107549	n-Hexadecanoic acid
5	18.899	16.49	127648	9,12-Octadecadienoic acid,
6	18.959	15.12	129353	9-Octadecanoic acid
7	19.149	5.95	131262	Octadecanoic acid
8	21.741	1.52	94142	Cyclopentadecanone,
9	23.418	1.60	115866	9-OctadecenaL
10	27.848	2.31	217434	gamma-Sitosterol

CONCLUSION

The study concludes that ethyl acetate, methanol, and aqueous extracts of *T. chebula* fruit were inhibitory to *P. gingivalis,* but ethyl acetate extract showed the lowest MIC value. The highest amount of polyphenol was demonstrated in methanol extract, whereas ethyl acetate extract showed the highest amount of terpenoids among the 5 phytochemicals quantified. GCMS analysis revealed the existence of 13 and 9 compounds, from methanol and ethyl acetate extracts, respectively. These compounds may enhance the antibacterial activity through a synergistic effect. These bioactive compounds should be isolated by HPLC and further evaluated for their antimicrobial and toxicological properties for use as a drug. These compounds after complete evaluation, can be incorporated into mouthwash, toothpaste, and topical dental

ointments. Not much literature is available on the activity of *T. chebula* fruit extract against *P. gingivalis*, hence, this study is significant.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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