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In vitro anti-lipid peroxidation and anti-*Candidal* potentials of different betel leaf varieties

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Abstract

Betel leaf (*Piper betel* L.) is an important plant being widely consumed worldwide as well as being used in traditional medicine. The present study aims to evaluate the anti-lipid peroxidation and anti-*Candida* potentials of different varieties of betel leaf extracts. Three widely consumed betel leaf varieties of India (Kolkata Variety, Varanasi Variety, and Mysore Variety) were used to evaluate anti-lipid peroxidation, and anti-*Candida* potentials in different extracts (ethyl acetate, acetone, and methanol). Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were found to be higher in the ethyl acetate extract of all the varieties and hence selected for medicinal potential evaluation. The Mysore variety exhibited higher antioxidant and free radical scavenging properties IC₅₀ value 179.90 ± 2.98 µg/mL and 319.52 ± 4.00 µg/mL respectively. The Varanasi variety with the IC₅₀ value of 51.54 ± 1.81 µg/mL has slightly high anti-lipid peroxidation potentials over the Mysore variety (IC₅₀ value 61.67 ± 2.92 µg/mL) over the standard butylated hydroxytoluene (BHT) with an IC₅₀ value of 276.33 ± 15.20 µg/mL. The oral lotion disinfectant formulation showed anti-*Candida* activity in a dose-dependent fashion and the activity was higher than that of the standard drug fluconazole with the inhibition zone of 15mm over fluconazole having zone of inhibition of 12mm. The present study confirmed that the antioxidant and anti-lipid peroxidation potentials of the ethyl acetate extract of the Mysore and Varanasi variety respectively. The study also confirmed the anti-*Candida* activity of the ethyl acetate extract of betel leaf.

Keywords: Betel leaf, antioxidant, anti-lipid peroxidation, anti-*Candida*, oral disinfection formulations

Introduction

Betel Leaf (*Piper betel* L.) belonging to the family Piperaceae is a climbing vine with deep green to light green heart-shaped leaves. Although it is native to Malaysia, out of 100 total varieties found in the world, 40 varieties are cultivated only in India and 30 varieties in Bangladesh [1]. In India, the cultivation of betel leaf takes place mainly in West Bengal, Uttar Pradesh, Karnataka, Orissa, Maharashtra, Tamil Nadu, and Madhya Pradesh. Apart from India, it is cultivated in countries like Bangladesh, The Philippines Islands, Myanmar, Malaysia, and Sri Lanka. Leaves, stalk, and stems of betel leaves are consumed. Leaves of betel leaf are consumed as betel quid and as mouth fresheners because of their sweet taste with strong flavors. Around 200 - 600 million people consume betel quid worldwide [2]. In the Indian culture, the betel leaf offered along with the Areca nut is especially important and represented as an auspicious symbol on various social and religious occasions [3].

It is also used in traditional medicine for various properties as a rich source of Calcium, anti-microbial, anti-fertility, anti-diabetic, cardio-tonic, and respiratory depressant [1, 4]. It is also rich in the Phyto-compounds such as hydroxychavicol, allyl pyrocatechol, eugenol, chavicol, etc., which attribute for the various properties like anti-mutagen, anti-tumor, anti-inflammatory, anti-oxidant, radioprotectant, lymphoproliferative agent, free radical scavenger and prevents platelet aggregation, [2, 4-6].

The essential oil extracted from the *P. betel* L. is known to have fungicidal effects against a wide range of fungal species including *Candida spp.*, *Aspergillus spp.*, and dermatophytes. *Candida spp.* is one of the most common fungal species present in the oral cavity being commensal and an opportunistic pathogen causing Candidiasis when the host immune system weakens [7].

These fungal pathogens are also associated with the formation of biofilms and can adversely affect the medical implants by forming a layer of biofilms on the surface of implants causing complete failure of the devices as well as providing the source for future infections [8]. Lipid peroxidation is a process of oxidative damage of the lipid especially the polyunsaturated fatty acids at carbon-carbon double bond(s). The process involves the removal of hydrogen and the addition of oxygen, resulting in the formation of Lipid peroxyl radical (LPR) and hydroperoxides [9]. A high level of free radicals or ROS in cells can also directly lead to lipid peroxidation. In response to the lipid peroxidation, at a low rate, cells stimulate the defense signaling involving the activation and upregulation of antioxidants resulting in adaptive stress response, while at a higher rate, cells undergo apoptosis or necrosis and eventually damage the cell and accelerate aging [10]. Many diseases like cancer, acute lung injury, diabetes, Alzheimer's disease, and Parkinson's disease are associated with lipid peroxidation [11]. The current study focuses on the *in vitro* anti-lipid peroxidation potentials and anti-*Candida* properties for the oral disinfection formulations of three selected varieties of *Piper betel* L. namely Mysuru Chigurele (Mysuru Variety), Banarsi Paan (Varanasi Variety), and Kolkata Paan (Kolkata Variety) extracts in different solvents namely ethyl acetate, acetone, and methanol.

Materials and Methods

Chemicals and Reagents

Folin-Ciocalteu's Reagent, Sodium Carbonate, Ferric Chloride, Ferrous Sulphate, and Acetic Acid were bought from Fisher Scientific. Gallic Acid, Potassium Acetate, Potassium Persulfate, Sodium Dodecyl Sulfate (SDS), and Thio-barbituric acid were bought from Himedia. Quercetin, Tri-chloroacetic Acid, 2, 2-diphenyl-1-picrylhydrazyl, 2, 2-Azo-Bis (3-Ethyl Benzothiazoline-6-Sulfonic Acid), and Methanol was bought from CDH chemicals. Butylated Hydroxytoluene (BHT) Potassium ferrocyanide was acquired from Rankem chemicals. Potato Dextrose Agar (PDA) media was prepared using 100g of potato infusion, 20g of dextrose (Himedia), and 15g of agar (Himedia) in 1000mL of distilled water for anti-*Candidal* assay. All chemicals were of high purity and analytical grade.

Sample Collection and Preparation

Fresh leaves of *Piper betel* L. were collected from the local market of Mysuru (Karnataka), Lucknow (Uttar Pradesh), and Kolkata (West Bengal) and were washed with tap water and distilled water and dried at room temperature.

Dried leaves were mechanically ground to form a powder and subjected to sequential solvent extraction in Soxhlet apparatus using ethyl acetate, acetone, and methanol. The extracts were concentrated by the solvent evaporation technique using a rotary evaporator (Heidolph, Germany). The extracts were used for carrying out various assays.

Phytochemical Analysis

Total Phenolic Content (TPC) Estimation

The total phenolic content of different extracts namely ethyl acetate, acetone, and methanol of different betel leaf varieties was determined using Folin-Ciocalteu (FC) reagent method [12]. Briefly, 250µl of 1:1000 diluted samples were added to 500µl of distilled water. 1.25 mL of 1:10 diluted FC was added and incubated for three minutes at room temperature. 1mL sodium carbonate (1mM) was added and incubated for two hours at room temperature and absorbance was measured at 760nm. Gallic acid was used as standard and the concentration was expressed as gallic acid equivalent (GAE)

(mg/g of dry mass).

Total flavonoid content (TFC) estimation

The total flavonoid content was determined by the aluminum chloride method [13]. Briefly, 250µl of 1:1000 diluted sample was mixed with 750µl of methanol, 50µl aluminum chloride (10%), 50µl potassium acetate (1M), and 1.4mL of distilled water. The reaction mixture was incubated for one hour at room temperature and absorbance was measured at 415nm. Quercetin was used as standard and the concentration was expressed as quercetin equivalent (QE) (µg/100g of dry mass).

Antioxidant assay

Reducing power assay

The reducing power assay was carried out according to the protocol described by Shen *et al.*, [14] with modification. The crude plant extracts were dissolved in methanol (1mg/mL). Different concentration of methanolic samples (10 to 100µg/mL) was prepared in 500 µl of distilled water. 1.25mL of phosphate buffer (20mM, pH 7.4) and 1.25 mL of potassium ferrocyanide (10%) were added and incubated for 20 minutes at 50°C. After cooling, 1.25mL of trichloroacetic acid (10%) was added to stop the reaction. The reaction mixture was centrifuged at 3000 rpm for 10 minutes. 1.2mL of the upper layer was mixed with 1.25mL of distilled water and 250µl of ferric chloride solution (0.1%). Absorbance was measured at 700nm. Ascorbic acid was used as an standard for the assay.

2, 2 – Diphenyl-1-Picryl Hydrazyl Hydrate (DPPH) Assay

DPPH free radical scavenging property was assessed according to the protocol described by Sultanova *et al.* [15] with modifications. Different concentrations of crude extracts (25 to 500µg/mL) were prepared in 20µl of methanol. The methanolic samples were taken on a 96-well plate. To this 180µl DPPH (300µM) was added. The plate was incubated in the dark at 37°C for 30 minutes and absorbance was measured at 515nm. The half-maximal inhibitory concentration (IC₅₀) value was calculated depicting the concentration of the sample needed to scavenge 50% of DPPH free radicals. Ascorbic acid was used as positive control while respective solvent mixed with ethanol was used as a negative control. Ascorbic acid was used as a standard for the assay.

2, 2 – Azo-Bis (3-Ethyl Benzothiazoline-6-Sulfonic Acid) (ABTS) Assay

ABTS Assay was carried out according to the protocol described by Re *et al.* [16]. ABTS solution was prepared by dissolving ABTS in water to a concentration of 7mM. ABTS radical cation was generated by adding 2.45mM of potassium persulfate to the ABTS stock solution. The mixture was allowed to stand in dark at room temperature for 12-16 hours before use. The ABTS solution was diluted with 95% methanol to an absorbance of 0.70 ± 0.02 at 734 nm.

The sample was prepared by dissolving different concentrations of crude extract (10-100µg/mL) in methanol. Absorbance was measured by adding 1.0 mL of diluted ABTS solution to 10µl of the methanolic sample at 734nm. The IC₅₀ value was calculated depicting the concentration of the sample needed to scavenge 50% of ABTS radicals. Quercetin was used as a standard for the assay.

Thio-barbituric Acid Reactive Substance (TBARS) Assay

The extent of lipid peroxidation by crude extracts of different

betel leaf varieties was determined by the estimation of TBARS by standard assay with modification [17]. Lipid peroxidation was carried out using egg yolk homogenate. Briefly, 250 μ l of egg yolk homogenate (10% v/v) was added to the crude extracts with varying concentrations (20-50 μ g/mL) and the final volume was made up to 500 μ l with distilled water. 25 μ l of ferrous sulfate (0.5mM) was added and the reaction was incubated for 30 minutes at 37°C. After incubation, 750 μ l of acetic acid (20%), 750 μ l of TBA (0.8%), and 100 μ l of SDS (8.1%) was added and kept for incubation at 95°C for one hour. After cooling, 1mL of n-butanol was added. The mixture was centrifuged at 9000 rpm at 4°C for five minutes. The organic layer was separated and absorbance was measured at 532nm and the IC₅₀ value was calculated. BHT was used as a standard for the assay.

Preparation of betel leaf-based oral disinfectant formulations

Fresh leaves of betel leaf (Mysuru Chigurele) were collected from the local market of Mysuru (Karnataka, India) and were used for the oral disinfectant formulation study using the steam distillation method for obtaining essential oils, which is to be used as an active ingredient. Extraction and isolation of betel leaf oil were performed with 15 g of leaf according to the method described by Sharma *et al.* [18]. A clear yellow color essential oil was produced upon removal of water by the addition of anhydrous sodium sulfate and was used for mouth wash formulation. The extracted oil was used for the preparation of two different oral disinfection formulations i.e., liquid formulation and lotion formulation.

The liquid formulation contains 5% betel leaf essential oil extract, 0.15% sodium benzoate, benzoic acid, 1% sorbitol, and 93.5% sterile distilled water [19]. Suspension of the lotion formulation was made using 20% Arabic gum. The Arabic gum powder was added to the lukewarm water and mixed thoroughly, following that, 3% Polyethylene Glycol 400 (PEG) was used as a binder, and 5% betel leaf essential oil was added to the suspension. As a preservative, methylparaben was dissolved in a minimal amount of 70% ethanol and further, 60 mL of sterile distilled water was added to the components [20]. The components were then stirred continuously for another 15 minutes and were used for further studies.

The stability of the formulations was evaluated using the method described by Hadning *et al.* [20]. Organoleptic observations, pH change, homogeneity test, and adherence test were performed for the evaluation of the lotion formulation. The organoleptic examination consisted of noting the changes in shape, color, and odor for 30 days at room temperature (28 \pm 2°C).

Anti-Candida Assay

The anti-*Candida* assay was performed using the disc

diffusion method. The fungal isolate of *C. albicans* (MTCC no. 183) was collected from the culture collection of the Department of Studies in Biotechnology, University of Mysore, Mysuru, Karnataka, India. A loopful of the slant culture was inoculated into the PDA medium and incubated for 48-72 hours at 28°C. Liquid and Lotion formulation was applied to the discs which were impregnated onto the medium and were incubated for 48 hours at 28°C, and the inhibitory zone surrounding the disc was measured [21].

Minimum inhibitory concentration (MIC) determination

The MIC of an anti-microbial agent is the lowest concentration at which the microorganisms appear to be inhibited from growing, with the higher the efficiency of the compound, the lower the concentration is required. The MIC was measured using the Clinical and Laboratory Standards Institute's micro broth dilution method. The extract concentration was tested at 0.1–2 mg/mL. In each test tube, 0.1 mL of standardized inoculum ($1-2 \times 10^7$ colony-forming unit/mL) was added. For 48–72 hours, the tubes were incubated aerobically at 28°C. For each test sample, two controls were kept. The MIC was determined by comparing the diluted extract with the control tubes and determining the lowest concentration (highest dilution) at which signs of microbial growth were not detected (no turbidity) [45].

Statistical analysis

Assays were carried out in triplicates and respective results were expressed as mean \pm standard deviation. Further, the IC₅₀ of different anti-oxidant assays were analyzed using the analysis of variance (ANOVA) test for the least significant difference with $p < 0.0001$ as a level of significance. Tukey's method of analysis was used for the multiple comparisons at a 95% confidence interval (CI) with $p < 0.0001$ as a level of significance.

Results

Phytochemical analysis

TPC estimation

The total phenolic content of the selected betel leaf varieties is expressed as mg GAE/g of the dry mass of the sample. The standard curve (provided as supplementary data Supplementary Figure.1) was plotted with varying concentrations of Gallic acid. From the standard curve, ethyl acetate extract of Mysuru Chigurele showed the highest amount of total phenolics i.e., 267.72 ± 1.18 mg of GAE/g of dry mass, while the ethyl acetate extract of Kolkata Paan and Banarsi Paan showed the total phenolic content of 245.17 ± 2.75 , 260.80 ± 0.45 mg of GAE/g of dry mass respectively as shown in Figure 1. Ethyl acetate was found to be the best solvent for the extraction of phenolics when compared to acetone and methanol, significantly containing ($p < 0.0001$) higher phenolic content.

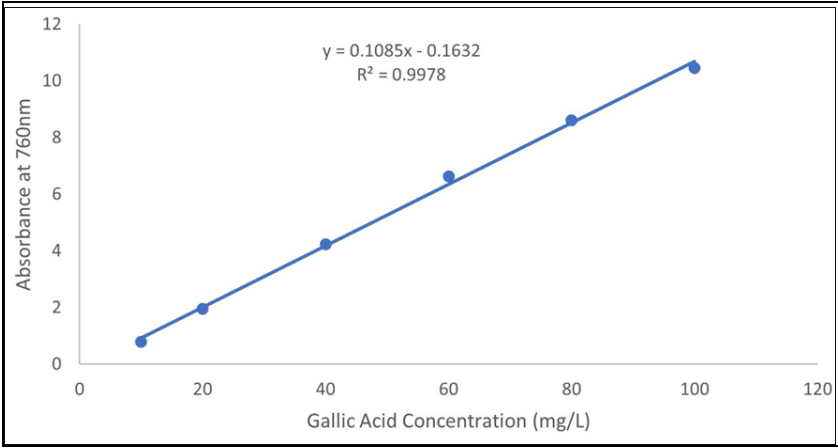


Fig 1: Standard Curve for the Total Phenolic Content (TPC) estimation of different selected varieties of betel leaf

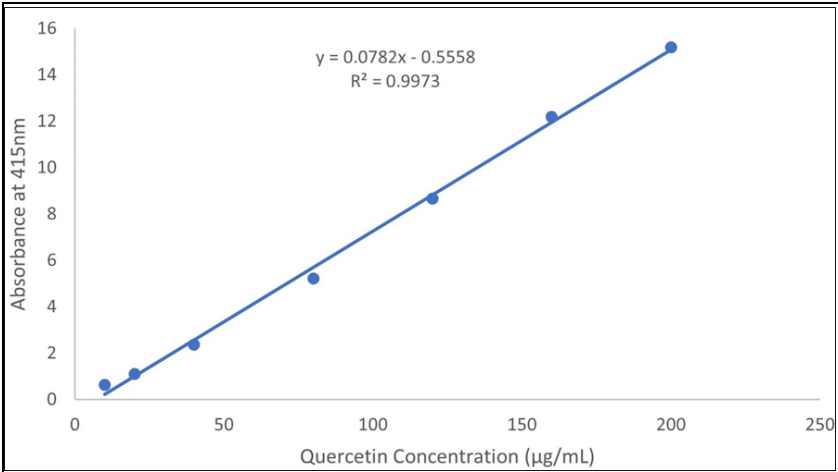


Fig 2: Standard Curve for the Total Flavonoid Content (TPC) estimation of different selected varieties of betel leaf

Table 1: Total Phenolic contents for Different Betle Leaf Varieties

Sample	Solvent		
	Ethyl Acetate	Acetone	Methanol
Kolkata Variety	167.843 ± 0.173	58.533 ± 2.671	73.990 ± 0.160
Varanasi Variety	171.815 ± 0.522	114.275 ± 2.907	67.488 ± 2.679
Mysore Variety	175.651 ± 0.981	131.598 ± 7.133	72.329 ± 0.294

Total Phenolics content (n=3), which is expressed as Gallic acid Equivalent (mg of Phenolics per gm of Dry mass).

Values in the table are represented as mean ± S.E (n=3)

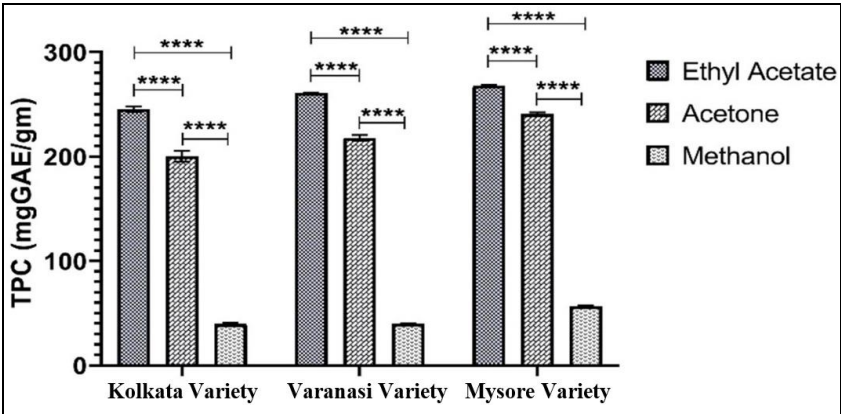


Fig 3: Total Phenolic Content (TPC) of different selected varieties of betel leaf in different selected solvent calculated based on the standard curve. Bars represent the Standard Deviation. **** represents a significant difference between different extracts within the same variety at $p<0.0001$

TFC Estimation
The total Flavonoid content of selected Betel Leaf varieties is

expressed as µg QE/100 g of the dry mass of the sample. The standard curve (provided as supplementary data

Supplementary Figure.2) was plotted with varying concentrations of quercetin. Total flavonoid content (TFC) of ethyl acetate extract of Kolkata Paan, Banarsi Paan, and Mysuru Chigurele total flavonoid contents was found to be 193.98 ± 0.56 , 199.249 ± 0.273 and 115.32 ± 0.39 $\mu\text{g QE}/100$ g of dry mass respectively as shown in Figure 2. The ethyl acetate extract of Banarsi Paan significantly ($p < 0.0001$) contains higher total flavonoid content. The other extracts i.e., acetone and methanol content had significantly ($p < 0.0001$) low total flavonoid content.

Table 2: Total Flavonoid content for different Betle Leaf Varieties

Sample	Solvent		
	Ethyl Acetate	Acetone	Methanol
Kolkata Paan	193.987 ± 0.566	21.632 ± 1.158	22.597 ± 0.158
Banarsi Paan	199.249 ± 0.273	31.767 ± 0.114	21.793 ± 0.097
Mysuru Chigurele	115.327 ± 0.394	63.416 ± 0.406	20.539 ± 0.255

Total Flavonoid content (n=3) expressed as Quercetin Equivalent (μg of Flavonoid per 100gm of Dry mass). Values in the table are represented as mean \pm S.E (n=3)

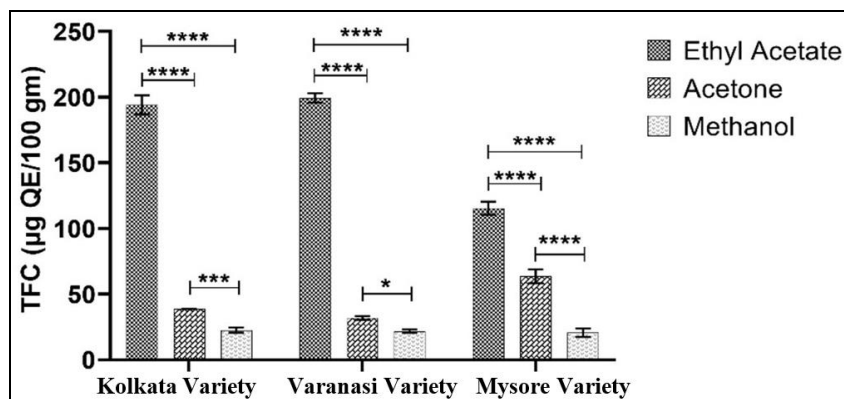


Fig 4: Total Flavonoid Content (TFC) of different selected varieties of betel leaf in different selected solvent calculated based on the standard curve. Bars represent the Standard Deviation. **** represents a significant difference between different extracts within the same variety at $p < 0.0001$

Antioxidant Assay

Reducing power assay

From the results, it can be deduced that the reducing ability of

the ethyl acetate extracts is in the order of Mysuru Chigurele > Banarasi Paan > Kolkata Paan over standard as shown in Fig 3.

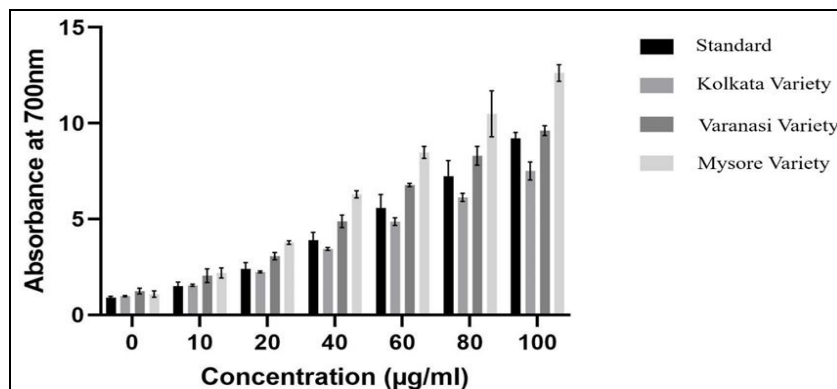


Fig 5: Reducing power assay of the ethyl acetate extracts of different varieties of betel leaf in comparison with the standard at different concentration, bar represents the standard deviation

DPPH Assay

DPPH free radical method is an anti-oxidant assay based on electron transfer that produces a violet solution in ethanol. The free radical remains stable at room temperature but gets reduced when an antioxidant molecule is present and gives a colorless ethanol solution. Lower the IC_{50} value, shows high free radical scavenging activity [23,24]. From the results, we can highlight that the antioxidant properties of ethyl acetate extracts are in the order of Mysuru Chigurele > Banarasi Paan

> Kolkata Paan. The IC_{50} was found to be 179.904 ± 2.987 $\mu\text{g/mL}$, 254.593 ± 2.139 $\mu\text{g/mL}$, and 418.682 ± 4.035 $\mu\text{g/mL}$ for Mysuru Chigurele, Banarasi Paan, and Kolkata Paan respectively, while the IC_{50} for the Standard Ascorbic Acid is 93.608 ± 5.488 $\mu\text{g/mL}$, as shown in Figure 4. The result shows that the Mysuru Chigurele has significantly ($p < 0.0001$) high DPPH free radical scavenging ability over other varieties of betel leaf.

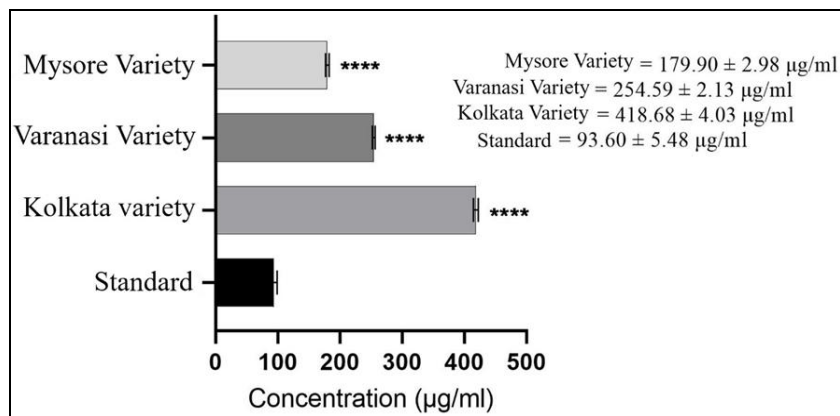


Fig 6: IC₅₀ values of the ethyl acetate extracts of different varieties of betel leaf in comparison with the standard. The value represents the mean ± SD based on triplicate, the bar represents the standard deviation, **** represents the significant difference between different varieties of betel leaf from the standard at $p < 0.0001$

ABTS assay

The ability of anti-oxidant to scavenge the ABTS radical which is generated in the aqueous phase is measured using ABTS assay, as compared with standard Quercetin. The ABTS radical is generated by reacting with a strong oxidizing agent (Potassium Persulfate) with the ABTS salt. Only ethyl acetate extract was showing inhibition. The IC₅₀ was found to

be 319.521 ± 4.009 µg/mL, 4152 ± 6.244 µg/mL, 4074.691 ± 8.090 µg/mL of Mysuru Chigurele, Banarsi Paan, and Kolkata Paan respectively as shown in Figure 5. The result shows that Mysuru Chigurele has significantly ($p < 0.0001$) high ABTS scavenging properties as compared to other varieties of betel leaf which is almost equal to the standard.

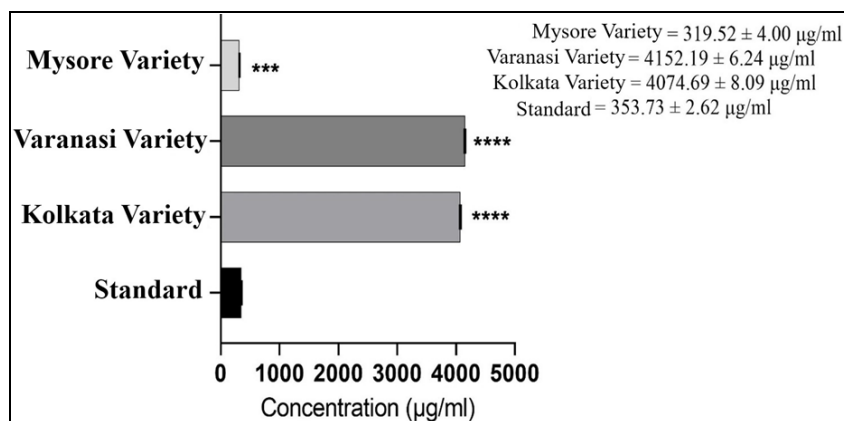


Fig 7: IC₅₀ values of the ethyl acetate extracts of different varieties of betel leaf in comparison with the standard. The value represents the mean ± SD based on triplicate, the bar represents the standard deviation, **** represents the significant difference between different varieties of betel leaf from the standard at $p < 0.0001$

TBARS Assay

The ethyl acetate extracts were found to inhibit FeSO₄-induced lipid peroxidation. Mysuru Chigurele and Banarsi Paan ethyl acetate extracts were significantly ($p < 0.0001$) effective in inhibiting lipid peroxidation. The IC₅₀ of ethyl acetate extract was found to be 65.677 ± 2.291, 51.546 ±

1.814, and 114.335 ± 4.708 µg/mL for Mysuru Chigurele, Banarsi Paan, and Kolkata Paan respectively as shown in Figure 6. The result shows that the ethyl acetate extracts of Banarsi Paan and Mysuru Chigurele have almost similar lipid peroxidation inhibition properties.

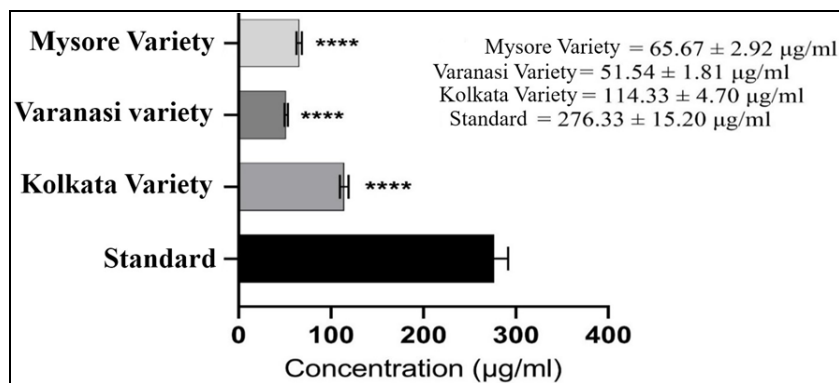


Fig 8: IC₅₀ values of the ethyl acetate extracts of different varieties of betel leaf in comparison with the standard. The value represents the mean ± SD based on triplicate, the bar represents the standard deviation, **** represents the significant difference between different varieties of betel leaf from the standard at $p < 0.0001$

Evaluation of betel leaf-based oral disinfectant formulations

According to organoleptic observations (as shown in Table 3), the concentration of Arabic gum comes within the framework of betel leaf formulation. The formulations were stored for 30 days to determine their stability properties. During storage, prepared oral liquid and lotion formulations maintain a continuous and stable balance in color, thickness, and odor, and there no major significant changes were observed. In the investigation, PEG was employed because of its properties like non-irritating, good adherence and distribution to the oral mucosa as well as it does not interfere with gas exchange and sweat production [25]

Prepared oral liquid formulation shows the soft texture, aromatic odor, and monophasic homogeneity form and after 30 days of incubation, the texture of the liquid changed to slightly rough, maintaining the same monophasic homogeneity with an aromatic odor. In lotion formulation, odor and monophasic form of homogeneity have been maintained persistently during the incubation period, and no significant changes were observed.

Based on pH observations, the pH in liquid and lotion formulations exhibit minimal variation, ranging from 4.7 to 5.1, this may be attributed because of the absence of any buffer system to synthetic formulations, resulting in modest pH change [20]. Homogeneity was one of the characteristics that determined the quality of the lotion formulation. The distribution of active components from betel leaf extracts in the lotion is influenced by homogeneity. The active components in the betel leaf extract must be diffused to provide effective anti-fungal efficacy. The homogeneity test

was carried out by smearing 2 X 2 cm of lotion formulation on a glass plate. After it was touched and rubbed. The lotion homogeneity test was determined by analyzing whether the lotion had been well mixed during the mixing process. Both liquid and lotion formulations had good homogeneity, which was well maintained after the storage period with a smooth homogeneous flow. Liquid formulation shows even monophasic for without any agglomeration and any sedimentation, a characteristic feature which has been maintained over incubation period under room temperature. After the incubation period, there were no major significant changes in the consistency of the liquid formulation, whereas, a slight variation of yellow color was observed with light sedimentation of finely divided solid at the bottom.

An adhesive test was done to evaluate the adhesive power of the lotion formulation on the oral mucosa, when it is applied, to assess the strength of the lotion formulation that could be linked to the different application regions, such as oral mucosa, and their ability to coat the mucosal surface in an impenetrable way without clogging pores or interfering with mucosal physical activities [26]. The length of the glass plates sticking or shifting after being rubbed with lotion was used to calculate the adhesive power. The lotion has less adhered to the applied oral mucosal region as a result of the shift, and it was smeared off. Lotion formulation showed complete stable and static stickiness with 2mm movement in one minute after 30 days of storage. The longer the storage period lasted, the faster the adhesion time would decrease. The stickiness profile of the lotion was taken every five days for 30 days in stored settings and it maintained the same tendency throughout.

Table 3: Organoleptic observations of synthesized oral disinfectant formulations

Organoleptic observations	Oral Formulation			
	Before incubation		After incubation period of 30 days	
	Liquid form	Lotion form	Liquid form	Lotion form
Color	White	White	Slightly yellowish	White
Odor	Aromatic	Aromatic	Aromatic	Aromatic
pH	4.2	4.5	5.1	4.9
Texture	Soft	Very soft	Slightly rough	Soft
Homogeneity	Monophasic form	Biphasic form composed of finely divided solids suspended in medium	Monophasic form	Biphasic form composed of finely divided solids suspended in medium
Appearance	Clear dispersion	Gloomy	Clear dispersion	Gloomy
Viscosity	Low	High	Low	High
Average of the adhesion test in minute	21	51	17	47

Anti-Candidal Activity

The anti-*Candidal* activity of betel leaf has been attributed to hydroxyl chavicol present in the extract. They modify the cell membrane structure, leading to the disruption of the microbial membrane structure [8,27].

The anti-*Candidal* activity of betel leaf extracted oil as well as the liquid and lotion formulations were compared with fluconazole, a standard drug. The inhibitory zone magnifies in a dose-dependent manner. The largest inhibitory zone was observed in 15% betel leaf oil, with 17mm, followed by 10% betel leaf oil, with 12mm, and fluconazole, with 12 mm (as shown in Table 4). The MIC of extracted betel leaf oil was determined at different concentrations (5%, 10%, and 15%). Fluconazole, a popular antifungal standard was used. Liquid and lotion formulations exhibit anti-*Candida* activity with 11mm and 15mm of a zone of inhibition respectively. Fluconazole had the lowest MIC value at 60µg/mL, followed by 5% betel leaf oil. Nanayakkara *et al.* [28] revealed that extracts from young betel leaves have stronger anti-*Candida*

activity than those of mature leaves. Resistant strains of *C. albicans*, a causative agent of oral candidiasis, have become a serious public health concern, further necessitating the development of innovative anti-fungal medicines to address the issue. These circumstances motivated researchers to look for new and effective antifungal medicines to replace the current regimens [29]. Previous research on betel leaf root and complete extract demonstrated potent anti-microbial action via secondary metabolite production [30, 31]. Hydroxychavicol, a significant phenolic component of betel leaf, has been shown to be antifungal [8]. Natural products are in high demand due to their comprehensive biological capabilities and bioactive compounds, which are effective against a wide range of disease-causing agents [32]. Studies on natural plant products such as betel and tulsi revealed that the secondary metabolites generated have potent antibacterial action [27, 33, 34]. In the current study, the oral disinfectant liquid was prepared with the aim to be used for the oral gargling to prevent and kill the frequently occurring infections in the mouth, and

lotion formulation was prepared with the aim to apply for the treatment of mouth ulcers. In the preliminary phase, it is tried to standardize the formulation and further study its anti-fungal activity in *in vitro* conditions. In future studies, the prepared formulation(s) will be used for the pre-clinical studies.

Table 2: Anti-*Candidal* activity of extracted betel leaf oil and oral disinfectant formulation

		Zone of inhibition (mm)
Betel leaf oil	5%	7
	10%	12
	15%	17
Oral disinfectant formulation	Liquid	11
	Lotion	15
Fluconazole		12

Discussion

The higher phenolic component is directly related to the higher antioxidant and free radical scavenging properties and oxidation of the phenols also leads to the protection of the plants from harmful microbes eliciting the anti-microbial properties [35].

The result is not in synchronous with total phenolic content, the possible justification may be given as the flavonoid content significantly varies due to genetic diversity, environmental stress conditions, and agricultural practices [36, 37]. Moreover, literature also suggests that the flavonoid content may not depend on the phenolic content [38] while only some flavonoids with specific molecular structures can act as a proton donor and show radical scavenging activity [39–41].

The reducing power assay method is based on the reduction potential of the substance, which reacts with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride forming a complex having an absorption maximum at 700nm. Higher absorbance may be attributed to higher reducing ability as well as potential antioxidant activity [42–44].

During lipid peroxidation, poly-unsaturated fatty acids present in the lipid membrane undergo oxidation resulting in the formation of malonaldehyde (MDA), which reacts with two molecules of TBA to form TBARS, a pinkish-red chromogen, read at 532 nm. The FeSO_4 induced lipid peroxidation in the egg homogenate and its inhibition by bioactive extracts were evaluated by TBARS assay.

Conclusions

Betel Leaf is used extensively as 'Paan' in different parts of India and there is a preference for betel leaf. Mysuru Chigurele being the GI-tagged variety from Karnataka, India, has long been neglected even though it possesses high medicinal values. The study has been carried out to fill the lacuna in the availability of the literature regarding the bioactive potentials of the Mysuru Chigurele, which is exclusively grown in the Mysuru district of Karnataka, India. The current study revealed that the Mysuru Chigurele (Mysuru Variety) has high phenolic content than the other varieties of the betel leaf i.e., Banarsi Paan (Varanasi Variety) and Kolkata Paan (Kolkata variety). It has a high anti-oxidant and free radical scavenging properties, which is the primary mechanism for the lipid peroxidation inhibition and can act as a potential target for the therapeutical applications as compared to the other popular varieties of the *Piper betel* L. and may decrease the need for the dependency on the chemically manufactured medicine, which has a majority

share in the market, up to a certain extent. The findings also show that the ethyl acetate extract of *P. betel* is more effective as an anti-*Candidal* agent against *Candida albicans*. By promoting these traditional medicines, we can help to replace synthetic drugs to which *Candida* has developed resistance. Furthermore, applying this plant product to pre-clinical conditions for the treatment of mouth ulcers and oral disinfectants can be effectively implied in the future, increasing the efficacy of this plant product compared to standard drugs.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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