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Phytochemical screening, antioxidant and antimicrobial activities of *Prunella vulgaris* for oral thrush

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ABSTRACT

Plant imitative products have been used for medicinal purposes for centuries. In traditional Indian medicine or Ayurveda, *Prunella Vulgaris* and many other herbs have been used as medicine. Traditional uses of plants have led to investigating their bioactive compounds, which have resulted in the detection of a significant number of therapeutic properties. The aim of present investigation was carried out to evaluate the phytochemical, antioxidant and antimicrobial activity of chloroform and hydroalcoholic leaves extracts of *Prunella Vulgaris* against microbial strains causing oral infections. Both chloroform and hydroalcoholic extracts revealed the presence of carbohydrate, triterpenoids/ steroids, flavonoids, tannin, phenolic compound and saponins were absent in only the chloroform extract. The bioactivities of the leaf extracts were qualified to their phytochemical constituents. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method and aluminium chloride method respectively. The *In vitro* antioxidant activity of chloroform and hydroalcoholic leaves extracts of *Prunella Vulgaris* was assessed against 2,2-diphenyl-1-picryl- hydrazyl (DPPH) radical scavenging activity, reducing power assay using standard protocols. The antimicrobial activity of chloroform and hydroalcoholic extracts of medicinal plants was evaluated using well diffusion method against *Escherichia coli* and *Candida albicans.* The TPC in chloroform extract was higher than that of the hydroalcoholic extract with concentration being 0.443 mg/g equivalent to gallic acid. The TFC in hydroalcohoilc extract was higher than that of the chloroform extract with concentration being 0.358 mg/g equivalent to rutin. The present study recognized leaves extract of *Prunella vulgaris* as a promising antioxidant and antimicrobial agent. However, further investigations are needed to understand the mechanistic basis of this effect of the extract and its chemical constituents thereof.

Keywords: *Prunella vulgaris*, Phytochemical, Antioxidant, Antimicrobial activity, Folins Ciocalteau reagent, Quantitative analysis

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INTRODUCTION

Biological activity is the origin for traditional medicine, which uses the pharmacological effectiveness of natural compounds present in herbal preparations for treating human diseases[1]. Plants constitute a good source of cheap and affordable drugs and medicinal plants possess therapeutic efficacy like their traditional drugs counterpart, yet they show little or less side effects[2]. Plants and their parts such as roots, stems, barks, leaves, flowers, fruits, seeds and exudates form an important major ingredient of drugs used in traditional herbal medicinal systems. The therapeutic competence of the drugs used in these systems really depends on the use of proper and authentic raw materials[3]. The screening of medicinal plant extracts and plant products for antimicrobial and antioxidant properties show that many of such plants are primary sources of antibiotics[4]. Native

groups have used curing plants as their personal phytomedical remedies[5]. To control human diseases antioxidant effects play an important role. Reactive oxygen species (ROS) related to lipid peroxidation is responsible for most of the pathogenesis[6]. Antioxidants provide confrontation against the oxidative stress by scavenging free radicals. Antioxidant activity is one of the most significant properties of plant extracts, because scientists have looked for sources of natural antioxidants to be introduced in many cosmetic, pharmaceutical and food formulations. The research for the new sources of antioxidants in the past resulted in the wide studies on medicinal plants[7]. *Candida* is a fungal pathogen[8] which are mostly known to cause high rate of mycotic infection to human worldwide[9]. *Candida* is known to cause mucosal and deep tissue infections. *Candida* infects mucosal tissues including mouth, esophagus, gut and vagina[10]. Oral candidal infections are considered opportunistic and its incidence has been increased remarkably since the widespread use of antibiotics and also in denture wearers, diabetics, HIV infected individuals, patients under chemotherapy and transplant recipients[11-13]. Even though several effective antifungal agents are available for oral candidal infections[14], the failure is not uncommon because isolates of *C. Albicans* may exhibits primary or secondary resistance to the drug during therapy[13]. To manage with the wide-spread problem of antimicrobial resistance, antimicrobial alternatives have been proposed[15-17]. Use of natural products for the control of fungal diseases is considered as an interesting option to synthetic fungicides due to their lower negative impact, reduced cost and adverse reactions to plant preparations compared to modern conventional pharmaceuticals. India is considered to be a rich emporium of drug plants, mainly used in preventive and curative medicine[18]. *Prunella vulgaris* L. (Lamiaceae), a plant known as self heal, was popular in traditional European medicine during the 17th century as a remedy for alleviating sore throat, reducing fever and accelerating wound healing. In China it was employed in folk medicine as a traditional antipyretic remedy[19, 20]. More recently, this plant has been used in the form of a hot water infusion to treat sores in the mouth and throat and as a crude aqueous extract in the clinical treatment of herpetic keratitis[21]. Flavonoids, as its active element, have important pharmacological actions and healthcare functions, which have a wide range of uses in the pharmaceutical, food, household chemical and other related industries. *Prunella vulgaris* L mainly contains triterpenoids and their glycosides, flavonoids, sterols and their glycosides, coumarin, organic acids, volatile oils, saccharides, etc [22-24]. In Europe, however, *P. vulgaris* is not classified as a medicinal plant at present. Lamaison *et al.*[25] found rosmarinic acid was the major phenolic component of this plant. *P. vulgaris* extract can be divided into polar and organic fractions. The polar fraction has been extensively studied with respect to biological (mainly antiviral) activity. Prunelline, the main component of this fraction exhibits anti-HIV activity[26-29] and prunellin also displays specific activity against the herpes simplex virus type 1 and 2[21]. It protects rat erythrocytes against haemolysis and kidney and brain homogenates against lipid peroxidation[30]. *P. vulgaris* aqueous-ethanol extract has also been shown to exhibit scavenger effects on DPPH[25]. The aim of this research was to evaluate the total phenolic and flavonoid contents of chloroform and hydroalcoholic leaves extracts of *Prunella Vulgaris and* their antioxidant, antimicrobial activities against different kind of microorganisms.

MATERIALS AND METHODS

Plant material

The leaves of *Prunella vulgaris* were collected from local area of Bhopal (M.P.) in the month of January, 2018.The sample was identified by senior Botanist Dr. Zia-Ul-Hassan, Professor and head of department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *Prunella vulgaris is* 119/Bot/Saf/18.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Animals

All ethical and handling guidelines were followed as set by Indian Legislation and approved by Institutional Animal Ethics Committee. All animals were procured and housed in animal house maintained under standard hygienic conditions. Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1824/PO/ERe/S/15/CPCSEA). Protocol Approval Reference No. PBRI/IAEC/PN- 17057.

Grouping of animals

Animals were housed in a group of five in separate cages under controlled conditions of temperature (22 ± 2°C). All animals were given standard diet (Golden feed, New Delhi) and water, *ad libitum.* The environment was also regulated at 25 ± 1^{0}C with 12/12 h (light/dark) cycle. Animals were further divided in four groups with six animals in each group. Group I: Control, Group II: Standard (Amphotericin B 5mg/kg), Group III: extract of *Prunella vulgaris* (200 mg/kg), Group IV: extract of *Prunella vulgaris* (400 mg/kg).

Biostatistical interpretation

All data are presented in Mean ±SD. Data were analyzed by One Way ANOVA followed by Bonferroni test. Values $P<0.05$ was considered as level of significance (n=4).

Extraction of plant material

Cold maceration

Leaves of *Prunella vulgaris* were collected, washed and rinsed properly. They were dried in shade and powdered mechanically. About 1kg of the Powder leaves was successive extracted with different organic solvents viz;

Chloroform and 70 % Methanol (Hydroalcohol) and allow to stored for 72 hours in ice cold condition for the extraction of phytochemicals. At the end of the third day extract was filtered using whatmann No. 1 filter paper to remove all un-extractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts[31].

Qualitative analysis of phytochemicals

The extracts prepared for the study were subjected to preliminary phytochemical screening by using different reagents for identifying the presence or absence of various phytoconstituents viz., carbohydrates, proteins, alkaloids, tannins, steroid, flavonoids and terpenoids in various extracts of medicinal plants. The above phytoconstituents were tested as per the standard method[32].

Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Chloroform and hydroalcoholic leaves extracts of *Prunella Vulgaris* are subjected to estimate the presence of TPC and TFC by standard procedure.

Total phenolic content estimation

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentration of (20-100 µg/ml) of gallic acid was prepared in methanol. Concentration of 100 µg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2 ml of a 10 fold dilute folin Ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and it was then Incubated at room temperature for 30 mins with intermittent shaking and the absorbance were taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve of Gallic acid and the results were expressed as gallic acid equivalent (mg/g) [33].

Total flavonoid content estimation

Different concentration of rutin (20 to 100 µg/ml) was prepared in methanol. Test sample of near about same polarity (100 µg/ml) were prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 min, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5min, and then 2 mL of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 mins. Absorbance was determined at 510 nm against water as blank. Total Flavonoid content was calculated by the Standard regression curve of Rutin/ Quercetin[34].

Antioxidant Activity

DPPH radical scavenging activity

For DPPH assay, the method of Gulçin *et al.*, 2006 [35] was adopted. A solution of 0.1mM DPPH (4mg/100ml) in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *Prunella Vulgaris* extracts. The change in colour was measured at 517 nm wavelength using methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

Reducing power assay

A spectrophotometric method was used for the measurement of reducing power. For this 0.5 ml of each of the extracts was mixed with 0.5ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferricyanide (10 mg/ml). The reaction mixture was incubated at 50 ºC for 20 min separately, and then rapidly cooled, mixed with 1.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (0.5ml) of the supernatant was diluted with distilled water (0.5ml) and then ferric chloride (0.5ml, 0.1%) was added and allowed to stand for 10 min. the absorbance was read spectrophotometrically at 700 nm. Ascorbic acid (AA) was used as standard for construction of calibration curve[36].

$$\textbf{Reducing Power (\%)} = (As / Ac) \times 100$$

Here, Ac is the absorbance of control (AA) and As is the absorbance of samples (extracts) or standards.

Acute oral toxicity

Acute toxicity study of the prepared leaves extracts was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423 [37] the animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, The dose level to be used as the starting dose is selected from one of three fixed levels 5, 300 and 2000 mg/kg body weight. Acute toxicity was determined as per reported method[37].

Oral Candidiasis

Preparation of extract solution

For oral candidiasis study, 200 and 400 mg/kg doses of hydroalcoholic plant extract were assayed against

Candida albicans induced oral candidiasis. Mice were divided into four groups of six animals in each group

A. **Vehicle treated with *Escherichia coli* treated group:** *Candida albicans* (3×10^8 CFU/ml) were dissolved in normal saline and were administrated by oral route at a dose of 10ml/kg body weight.
B. **Standard drug treated group:** Amphotericin B was dissolved in normal saline and was administrated by oral route at a dose of 5mg/kg body weight.
C. **200 mg/kg extract treated group:** Hydroalcoholic extract was dissolved in normal saline and was administrated by oral route at a dose of 200m g/kg body weight.
D. **400 mg/kg extract treated group:** Hydroalcoholic extract were dissolved in normal saline and was administrated by oral route at a dose of 400mg/kg body weight.

Organisms and inoculum preparation

Candida albicans will be cultured. The culture will be harvested by centrifugation at 2500rpm and then cells will be held three times in phosphate buffer saline (PBS) and adjusted to a final concentration of $3x10^8$ CFU/mL (using a Spectrophotometer for counting cells).

Oral candidiasis in the rat

To enhance the infection rate, rats were pretreated with extract and Amphotericin B (standard drug) for 7 days and on 6 day 0.1mL of saline suspension containing 3×10^8 viable cells of *C. albicans* injected intra-peritoneally before the drug and dosing were continue for next day.

Oral infection will be achieved by means of a cotton swab rolled twice over all parts of the mouth. Just before inoculation, the animals will be sampled to confirm the absence of *C. albicans* in the oral cavity and 72 h after the last inoculation all groups will be sampled in the same manner to check for the presence of the fungi and to quantify the number of CFU in the oral cavity before the beginning of the treatment.

Microbiology

Samples will be collected at days: 8 (i.e. 24 hours after the last treatment) by rolling a sterile cotton swab over the oral cavity, which will be then suspended in 1 mL of sterile saline. 25µl samples from this suspension will be dropped in duplicate, after serial tenfold dilution on Sabouraud agar plates containing 0.05% chloramphenicol. All plates will be incubated at 30°C for 24 h, and the colonies will be counted. The number of viable cells will be determined using the drop count method to calculate the log of the CFU/ml[38].

RESULT AND DISCUSSION

Phytochemical analysis of chloroform and hydroalcoholic extracts of leaf of *Prunella Vulgaris* showed the presence of carbohydrate, flavonoids, phenolics, tannin, saponins, triterpenoids table 1.

Table 1: Result of phytochemical screening of *Prunella Vulgaris* L.

Test	Hydroalcohalic	Chloroform
Test for carbohydrates		
Molish	+ve	+ve
Fehling's	-ve	+ve
Benedict's	+ve	-ve
Test for protein and amino acid		
Biuret	-ve	-ve
Ninhydrin	-ve	-ve
Test for glycosides		
Borntrager's	-ve	-ve
Keller-killani	-ve	-ve
Test for alkaloids		
Mayer's	-ve	-ve
Hager's	-ve	+ve
Wagner's	-ve	-ve
Test for saponins		
Froth test	+ve	-ve
Test for flavonoids		
Lead acetate	+ve	+ve
Alkaline reagent	+ve	+ve
Test for triterpenoids and steroids		
Salkowski's	+ve	+ve
Libermann-burchard's	+ve	+ve
Test for tannin and phenolic compounds		
Ferric chloride	+ve	-ve
Lead acetate	+ve	+ve
Gelatin	-ve	+ve

Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and the TPC in hydroalcoholic extract was found to be 0.361 mg/g equivalent to gallic acid while the TPC was higher in the chloroform extract, the concentration was 0.443mg/g table 2 & fig 1.

Table 2: Total phenolic content of extracts

S. No	Absorbance	
	Hydroalcoholic	Chloroform
1	0.794	0.307
2	0.794	0.306
3	0.791	0.305
4	0.791	0.305
5	0.793	0.305
TPC	0.361 mg/gm equivalent to Gallic acid	0.443 mg/gm equivalent to Gallic acid

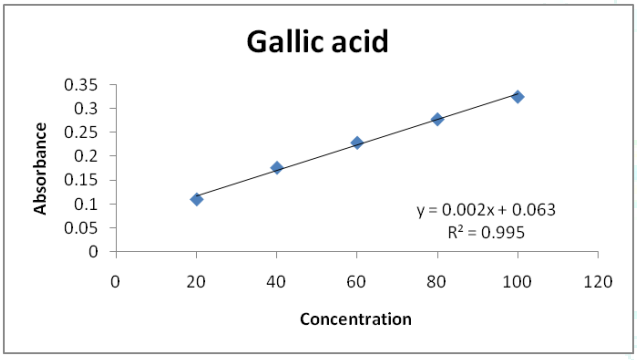

Figure 1: Graph of estimation of total phenolic content

TFC was then calculated with respect to rutin taken as standard. The TFC in hydroalcohoilc extract was higher than that of the chloroform extract with concentration being 0.358 mg/g equivalent to rutin table 3 & fig 2.

Table 3: Total flavonoid content of extracts

S. No	Asorbance	
	Hydroalcoholic	Chloroform
1	0.794	0.307
2	0.794	0.306
3	0.791	0.305
4	0.791	0.305
5	0.793	0.305
TFC	0.358 mg/gm equivalent to Rutin	0.213 mg/gm equivalent to Rutin

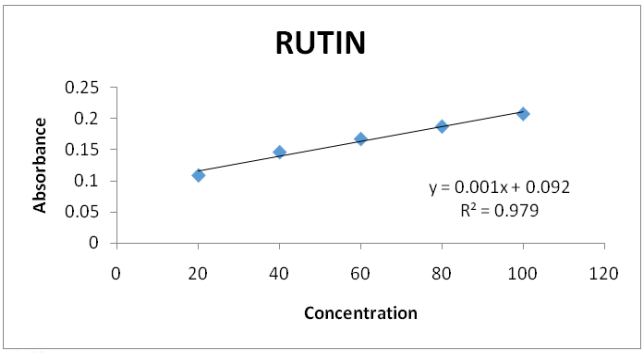

Figure 2: Graph of estimation of total flavonoid content

Antioxidant activity of the samples was calculated through DPPH assay and reducing power assay. % inhibition was calculated as an indicative of antioxidant potency. The higher the % inhibition the better the activity. Ascorbic acid was taken as standard in both the tests and the values were comparable with concentration ranging from 20µg/ml to 100µg/ml. A dose dependent activity with respect to concentration was observed. In DPPH assay % inhibition was higher in the hydroalcoholic extract where % inhibition ranged from 38.26754 % to 50.21930 % while the values were lesser in chloroform extract ranging from 33.77193 % to 42.32456 % table 4.

Table 4: DPPH assay of ascorbic acid, chloroform extract, hydroalcoholic extract

S. No.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	Chloroform extract (% Inhibition)	Hydroalcoholic extract (% Inhibition)
1.	**20**	52.74123	33.77193	38.26754
2.	**40**	56.35965	36.95175	40.35088
3.	**60**	61.51316	37.39035	43.85965
4.	**80**	68.9693	39.36404	46.60088
5.	**100**	71.71053	42.32456	50.21930

The reducing ability of the compound usually depends on the reductants, which have been exhibited antioxidative capacity by breaking the free radical chain, donating a hydrogen atom. Reducing power assay was calculated in both the extracts and the values indicated a better activity in hydroalcoholic extract than the chloroform extract table 5 & fig 3.

Table 5 Result of reducing power assay

Conc. (µg/ml)	Ascorbic acid	Hydroalcoholic extract	Chloroform extract
20	0.987	0.222	0.065
40	1.032	0.245	0.078
60	1.145	0.269	0.098
80	1.159	0.286	0.112
100	1.196	0.296	0.129

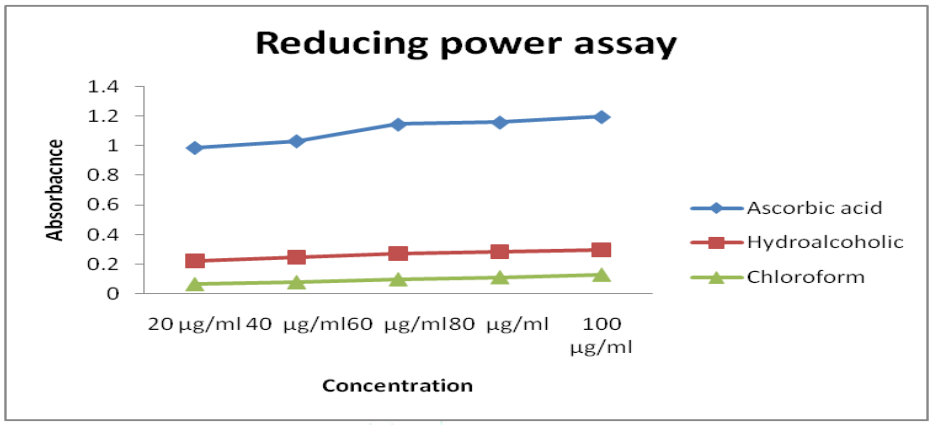

Figure 3: Reducing power assay

Table 6: Acute oral toxicity

S. No.	Groups	Observations/ Mortality
1.	5 mg/kg Bodyweight	0/3
2.	300 mg/kg Bodyweight	0/3
3.	2000 mg/kg Bodyweight	0/3

Acute oral toxicity was calculated at three different concentrations 5mg/kg, 300 mg/kg and 2000 mg/kg. Observations were performed in groups of three and no mortality was observed table 6.

Antimicrobial activity was calculated through well diffusion assay. The extract was given at different doses from 100 mg/ml to 250 mg/ml; better activity was exhibited by the hydroalcoholic extract and highest at the 250 mg/kg dose indicating a dose dependent activity table 7 & fig 4.

Table 7: Antimicrobial activity of *Prunella Vulgaris* extract against the bacterial strains tested based on well diffusion method

Concentration	100 mg/ml	150mg/ml	200 mg/ml	250 mg/ml
Hydroalcoholic Extract	**11.25±0.500**	**12.50±0.577**	**13.50±0.577**	**15.25±0.957**
Chloroform Extract	5.50±0.577	6.50±0.577	8.50±0.577	11.75±0.500

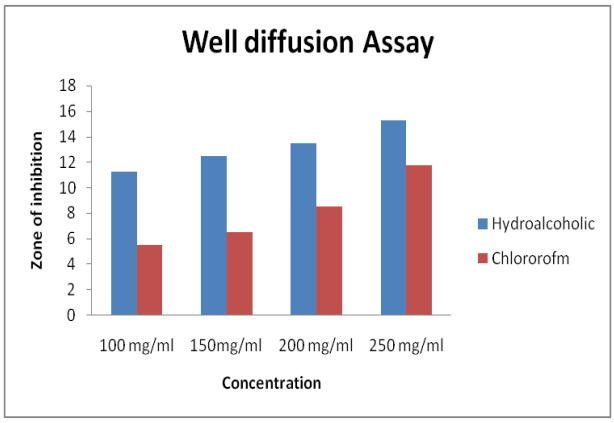

Figure 4: Antimicrobial activity of *Prunella Vulgaris*

Then the results were tested for the antimicrobial activity against the *candida albicans*. There was a great reduction in the CFU/ml found when the sample was treated with the extracts. At the extract dose 400 mg/kg the colony formation was lesser while the values were higher at the lesser dose of 200 mg/kg indicating a dose dependent table 8 & fig 5.

Table 8: Oral candidiasis activity

S. No.	Groups	Time in Seconds
1.	**Control**	339.00±15.875*
2.	**Standard**	160.33±14.012**
3.	**Ex (200 mg/kg)**	241.00±1.732*
4.	**Ex (400 mg/kg)**	204.33±16.042**

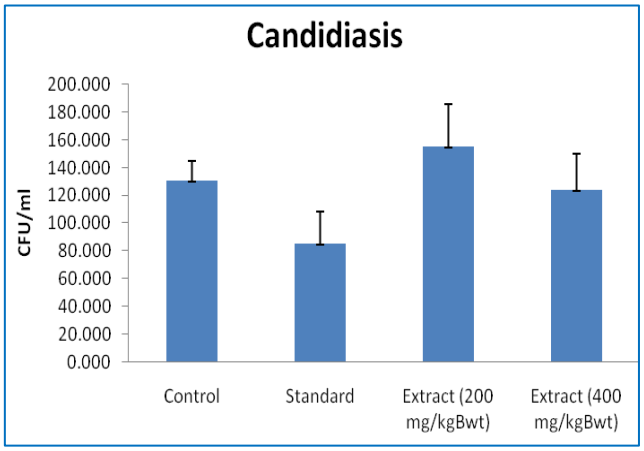

Figure 5: Oral candidiasis activity

CONCLUSION

The chloroform and hydroalcoholic leaves extracts of *Prunella Vulgaris* contains compounds with antimicrobial properties, which can be used as antimicrobial agents in pharmaceuticals and natural therapies of infectious diseases in humans, management of plant diseases and preservation and/ or extension of the shelf-life of raw and processed foods. The antioxidant activity of the *Prunella Vulgaris* extracts indicates that they have a protective effect against ROS and can therefore be used as a natural preservative ingredient in the food or pharmaceutical industry. The antioxidative activity observed in the *in vitro* cultured callus is particularly important, since the production of active principles can be provided throughout the year. These plants have potential for development of antimicrobial agents against oral microorganisms, for use in tooth paste, mouth wash etc for preventing and treating oral infections.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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