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ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL OF *MURRAYA KOENIGII* AGAINST HUMAN CARIOGENIC PATHOGENS

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ABSTRACT

This study was to evaluate the antioxidant and antibacterial efficacy of herbal crude extract of *Murraya koenigii* against human Cariogenic pathogens. In this study Methanolic extract of *Murraya koenigii* were studied for phytochemical composition and GC-MS for active compound. Antioxidant potential assay by 1,1-Diphenyl- 2-picrylhydrazyl (DPPH), 2, 2'-azinobis-3 ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) assays. Further extracts were studied for antibacterial activity. Methanolic extract of *Murraya koenigii* revealed the presence of terpenoids, alkaloids, tannins, steroids, and saponins. Reducing ability of extracts was in the range (in $\mu\text{m Fe(II)/g}$) of 3176.98 ± 63.87 were closed to quercetin 3208.27 ± 31.29 . A significant inhibitory effect of extract ($\text{IC}_{50} = 0.019 \pm 0.69 \text{ mg/ml}$) on ABTS free radicals was detected. The antioxidant activity of the extract (96%) DPPH radicals were comparable with that of ascorbic acid (98.8%) and BHT (97.5%). Antibacterial effect of 50% and 10% methanolic extracts was detected on Cariogenic pathogens as compared with standard drug Amoxicillin. GC-MS results revealed the presence of hexadecanoic acid, linoleic acid, oleic acid, propanoic acid, acetophenone. Extracts of *Murraya koenigii* contain significant amounts of phytochemicals, antioxidant and antibacterial property and it is exploited as a potential source for natural medicine.

Keywords:

Antioxidant,
Murraya Koenigii,
Dental caries,
Natural medicine

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INTRODUCTION: Dental caries is an infectious microbial disease that results in localized dissolution and destruction of the calcified tissues of the teeth¹. The untreated condition may lead to pain, tooth loss, infection and finally death in severe cases. Today, caries remains one of the most common diseases throughout the world. *Streptococcus mutans* is known as the causative bacteria in the formation of dental plaque and dental caries. The acid producing *S. mutans* causes damage by dissolving tooth structures in the presence of fermentable carbohydrates such as sucrose, fructose, and glucose².

The food debris, acid, bacteria, and saliva combine in the mouth to form a sticky substance called "plaque" that adheres to the teeth³. Dental disease is painful, and most importantly, it has also been suggestively linked to diabetes, high blood pressure, heart disease. The pain can be worsened by heat, cold or sweet foods and drinks^{4, 5}. Treatment often prevents further infection of the tooth structure. Early treatment is less painful than treatment of extensive decay. Dental caries can also cause bad breath and foul tastes. In highly progressed cases, infection can spread from the tooth to surrounding soft tissues which may lead to an

edentulous mouth⁶. Antibiotics such as Amoxicillin and erythromycin have been reported to effectively prevent dental caries in animals and humans, but they are never used clinically because of many adverse⁷.

Indian medicine is one of the oldest organized systems of medicine. Its earliest concepts are set out in the sacred writings called the *Vedas*, especially in the metrical passages of the *Atharvaveda* (2nd millennium BC). Recent natural remedies with the use of medicinal plants, which are good reservoirs of chemotherapeutants are being becoming as an alternative for antibiotic adverse effects such as hypersensitivity reaction, supra infections, and teeth staining^{8,9}.

The literature survey of the folklore medicine reveals the use of *Murraya koenigii* (Curry Tree) leaves to the treatment of infection, despite several anticaries agents being available commercially, the search for an effective natural agent still continues. Natural products have shown to be a good alternative to synthetic chemical substances for caries prevention¹⁰.

Knowing the fact that little literature is available on the use of *Murraya koenigii* in oral infection, the study is focused on assessing the *Murraya koenigii* extracts with different solvents. Hence, for the present investigation, *S. mutans*, *S. sanguinis*, *S. aureus*, *L. casei* are the bacterial strains selected as target organisms from patients and screened using methanolic extract of *Murraya koenigii* leaf. Once the antimicrobial property of the extracts is screened under *in vitro* condition against oral pathogens, *in vivo* trials can be carried out for the treatment of dental caries by external application on the caries tooth or as a preventive mouth rinse.

MATERIAL AND METHODS:

Microorganisms. The human dental caries pathogens, *Streptococcus mutans*, *Streptococcus sanguinis*, *Staphylococcus aureus*, *Lactobacillus casei* used in this study, were isolated from patients of the OPD's of Peoples Dental Academy, Bhopal, M.P., India.

Media Used. Thioglycolate Broth (TGB) and Brain Heart Infusion Broth (BHI) (Himedia laboratories, Mumbai India) are the transport media used to maintain clinical dental caries sample in viable condition.

Growth media used in examining of samples are Nutrient Agar (NA), Blood Agar (BA), Mutant Sanguis Agar, Manitol Salt Agar, de Man, Rogosa Sharpe Agar (MRS) (Himedia laboratories, Mumbai India) and Brain Heart Infusion Broth (BHI) are used for the antimicrobial susceptibility testing.

Collection and Recovery of Caries Sample. The samples from patients were collected under strict aseptic condition from the OPD of Peoples Dental Academy, Bhopal, M.P., India. Patient was made to rinse the tooth with water, and it was isolated with a rubber dam. The tooth and the surrounding field were cleaned with 3% hydrogen peroxide and then decontaminated with a 2.5% sodium hypochlorite solution. The food debris on the chewing surface were removed using a dental excavating instrument.

The dental caries sample was collected from the patient using an excavator under aseptic conditions by a clinician and was introduced into the 2ml broth of TGB or BHI in appropriate sterile screw cap bottles. The clinical samples were mixed well using a magnetic stirrer before incubation. The samples were then inoculated using the streak plate technique on to the appropriate culture media under various culture conditions and further identified by specific biochemical test.

Plant Materials Collection. We selected leafs of *Murraya koenigii* plant for antioxidant and antimicrobial assay, based on their ethnomedicinal and traditional uses against based on literature survey.

Preparation of Crude Extracts. The plant parts were shade-dried and powdered and used for extraction, 100 g of dry powder was taken in an aspirator bottle, 300mL Methanol (1: 3 W/V) was used and the mixture was shaken occasionally for 48 hour. Then, the extract was filtered. This procedure was repeated three times and all extracts were decanted and combined. The extracts were filtered before drying using Whatman filter paper no. 2 on a Buchner funnel, and the solvent was removed by vacuum distillation in a rotary evaporator at 40°C for quantitative determination; the extracts were placed in preweighed flasks before drying¹⁰.

Phytochemical Analysis: The presence of phytochemicals such as alkaloids, saponins, tannins, terpenoids (2, 4-dinitrophenyl hydrazine) and steroids (Liebermann–Burchard test) were evaluated according to the methods described by Edeoga *et al*¹¹.

***In-vitro* antioxidant assays:**

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay: The effect of extracts on DPPH radicals was estimated according to the method of Blois¹² with minor modifications. The methanolic extract was lyophilised and dilutions from 0.02 mg/ml

to 0.1 mg/ml prepared. One millilitre (0.135 mM) of DPPH solution was mixed with 1.0 ml of extract (in methanol). The reaction mixture was vortex-mixed thoroughly and incubated at room

temperature in the dark for 30 min. Reduction in the absorbance of the mixture was measured at 517 nm using ascorbic acid as a control. Scavenging of DPPH radicals by the extract was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{[(\text{Abscontrol} - \text{Abssample})]}{(\text{Abscontrol})} \times 100$$

Where Abscontrol is the absorbance of DPPH and Absample is the absorbance of the DPPH radical + sample extract/standard. The half maximal inhibitory concentration (IC50) values denoted the concentration of sample required to scavenge 50% of DPPH free radicals.

2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay ABTS solution (7mM) and 2.4 mM potassium persulfate (PPS) solution were mixed in equal volume and left to react for 12 h in the dark to prepare a working solution. One millilitre of a diluted working solution of ABTS–PPS was mixed with 1 ml of plant extracts, and the absorbance read at 734 nm after 7 min. ABTS.+ the scavenging capacity of the extract were compared with standard butylated hydroxytoluene (BHT)¹³. The percentage inhibition of the formation of ABTS.+ was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{[(\text{Abscontrol} - \text{Abssample})]}{(\text{Abscontrol})} \times 100$$

Where, Abscontrol is the absorbance of ABTS radical + methanol; Absample is the absorbance of ABTS radical + sample extract/standard.

Ferric Reducing Antioxidant Power (FRAP) Assay: A modified method of that used by Benzie and Strain was adopted for the FRAP assay¹⁴. A solution of 20 mM FeCl₃·6H₂O, 300 mM acetate buffer (3.1 g C₂H₃NaO₂·3H₂O in 16 ml C₂H₄O₂, pH 3.6) and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl) was prepared. At the time of establishing the assay, 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl₃·6H₂O was mixed to prepare the FRAP solution. Plant extract (150 μl) was mixed with 2850 μl of FRAP solution and incubated at room temperature in the dark for 30 min. Absorbance of the intense blue-coloured product (ferrous tripyridyltriazine complex) was measured at 593 nm. The observed absorbance of the sample was calculated by putting the values on a linear standard curve plotted between 200 μM to 1000 μM FeSO₄. Results were expressed in μM Fe(II)/g dry mass of methanolic extract of *Murraya koenigii*.

***In vitro* Anti bacterial Susceptibility Assay:**

- 1. Disc Diffusion Method-** Antimicrobial activity was carried out using disc-diffusion method¹⁵. Petri plates were prepared with 20mL of sterile brain heart infusion agar (BHI) for (Himedia, Mumbai) The test cultures (100 μL of suspension containing 10⁸ CFU/mL bacteria) were swabbed on the top of the solidified media and allowed to dry for 10min. The tests were conducted at three different concentrations of the crude extract (200 mg crude extract dissolved in 5% dimethyl sulfoxide (DMSO), respectively, 5mg and 2.5mg per disc). The sterile 6mm disc (Himedia) impregnated with different concentrations of extracts. The loaded discs were placed on the surface of the medium and left for 30min at room temperature for compound diffusion. Negative control was prepared using respective solvent. Amoxicillin (5mg/ml) were used as positive control. The plates were incubated for 24 h at 37°C. Zone of inhibition was recorded in millimeters and the experiment was repeated twice.
- 2. Minimum Inhibitory Concentration:** The extracts were dissolved in water +2% dimethyl sulfoxide

(DMSO). The initial concentration of extract was 5 mg/ml to 0.090 mg/ml. The initial test concentration was serially diluted two-fold. Each well was inoculated with 5 μ L of suspension containing 10^8 CFU/mL of bacteria. The antibacterial agent Amoxicilline include in the assays as positive controls. The plates with bacteria were incubated 24 h at 37°C. After incubation, 5 μ L of tested broth was placed on the sterile BHI plates and incubated at respective temperature. The MIC for bacteria was determined as the lowest concentration of the extracts inhibiting the visual growth of the test cultures on the agar plate. Triplicates were maintained¹⁶.

Gas Chromatography-Mass Spectrometry (GC-MS).

The active extracts were quantified using gas chromatograph (GC-MS-Shimadzu) equipped with a CPB-capillary column (mm inner diameter \times 50m length) mass spectrometer (ion source 200°C, RI 70 eV) programmed at 40°C–280°C with a rate of 4°C/min. Injector temperature was 280°C; carrier gas was He (20 psi).

RESULT:

Phytochemical analyses: Phytochemical screening of the methanol extracts of the *Murraya koenigii* showed the presence of alkaloids, tannins, steroids, glycoside and saponins (**Table 1**).

TABLE 1: PHYTOCHEMICAL ANALYSIS OF MURRAYA KOENIGII PLANT EXTRACT

Plant Extract	Alkaloids	Tannins	Flavanoids	steroids	glycosides	saponin
<i>Murraya Koenigii</i>	+	+	+	+	+	+

Key : - = Negative (absent), + = Positive (present)

In-vitro Antioxidant Activity:

Inhibition of DPPH Radicals: The DPPH method is widely used to measure the antioxidant properties of compounds. DPPH is organic nitrogen radical with ultraviolet–visible absorption in the range 515–520 nm, and the color of its solution fades upon reduction. The dose-response curve of DPPH radical scavenging activity of the methanolic extracts of the *Murraya koenigii* were compared with those of BHT and ascorbic acid. The *Murraya koenigii* extracts almost identical free-radical scavenging activity (96%) at 0.1 mg/ml. The scavenging activity of controls (ascorbic acid and BHT) was 98.9% and 97.6%, respectively. The IC₅₀ values obtained for *Murraya koenigii* extract were 0.028 \pm 0.44 mg/ml and for ascorbic acid and BHT were 0.015 \pm 0.58mg/ml and 0.028 \pm 0.31mg/ml, respectively (**figure 1**).

ABTS Radical Scavenging Assay: ABTS oxidized with PPS (absorption maxima at 734 nm) leads to the generation of ABTS free radicals; it is based on the ability of antioxidants to quench the ABTS + radical cation¹³. Methanol extracts of *Murraya koenigii* was rapid and effective scavengers of the ABTS radical and this activity was comparable with that of BHT. At 0.1 mg/ml, the percentage inhibition was 98% for BHT, 96% for *Murraya koenigii* extracts. The IC₅₀ value for BHT, *Murraya koenigii* were 0.013 \pm 0.32 mg/ml and

0.019 \pm 0.69 mg/ml respectively. The relative reducing power of all the extracts was in the order: BHT > *Murraya koenigii* extracts.

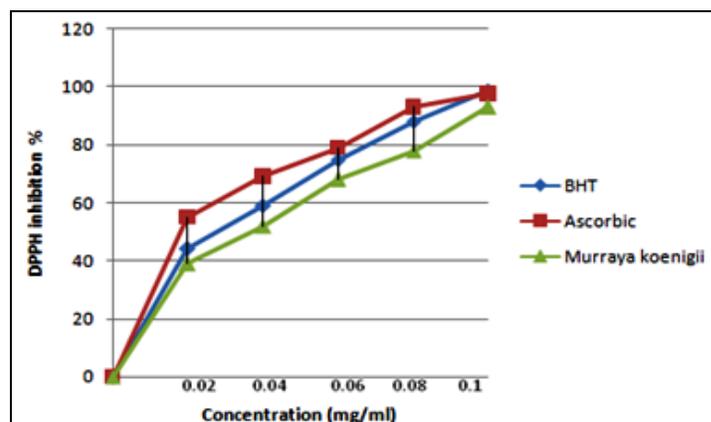


FIGURE 1: DPPH ACTIVITIES OF METHANOLIC EXTRACT OF MURRAYA KOENIGII

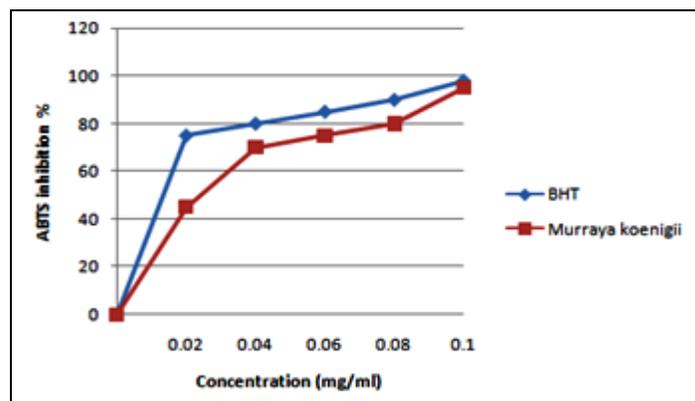


FIGURE 2: ABTS ACTIVITIES OF METHANOLIC EXTRACT OF MURRAYA KOENIGII

FRAP Assay: This assay can be used to assess the antioxidant potential in the extract of *Murraya koenigii* by showing their ability to reduce the TPTZ–Fe(III) complex to TPTZ–Fe(II). The reducing ability of *Murraya koenigii* extracts ($3176.98 \pm 63.87 \mu\text{m Fe (II)/g}$) were close to that of quercetin ($3208.27 \pm 31.29 \mu\text{m Fe(II)/g}$), which is the most researched type of flavonoid.

In vitro Antimicrobial Assay: The antibacterial activity of *Murraya Koenigii* is shown in **Table 2**. The antibacterial efficacy of extracts 50% Methanol, 10% Methanol, of the *Murraya Koenigii* plants against the human pathogenic bacteria showed varied level of inhibition. The activity was compared with standard drug Amoxicillin. Activity of different solvent extract of the *Murraya Koenigii* was assessed by disc diffusion method and well diffusion method. The antimicrobial activity of the *Murraya Koenigii* extract was found to be most potent antimicrobial agent. As per result,

TABLE 2: ANTIBACTERIAL ACTIVITY OF MURRAYA KOENIGII

Name of oral pathogens	<i>Murraya koenigii</i> extract (Zone of Inhibition of 15 μl extract in mm)				
	Amoxicilline	50% Methanolic		10% methanolic	
	5mg/ml	2.5mg/ml	5mg/ml	2.5mg/ml	5mg/ml
<i>Streptococcus mutans</i>	12	26	31	21	26
<i>Streptococcus sanguinis</i>	14	24	27	20	22
<i>Staphylococcus aurues</i>	12	19	28	21	23
<i>Lactobacillus acidophilus</i>	13	20	26	24	26
<i>Lactobacillus casie</i>	11	21	28	22	24

TABLE 3: MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF MURRAYA KOENIGII BY MICROBROTH DILUTION METHOD

Name of oral pathogens	<i>Murraya koenigii</i> extract	
	(Minimum inhibitory concentration mg/mL)	
	50% Methanolic	10% methanolic
<i>Streptococcus mutans</i>	0.090	0.090
<i>Streptococcus sanguinis</i>	0.090	0.090
<i>Staphylococcus aurues</i>	0.360	0.72
<i>Lactobacillus acidophilus</i>	0.090	0.72
<i>Lactobacillus casie</i>	0.090	0.360

Gas Chromatography-Mass Spectrometry (GC-MS)- In this study, the extract of *Murraya Koenigii* were subjected to active chemical component evaluation, GC–MS analyses revealed the presence of hexadecanoic acid, linoleic acid, oleic acid, propanoic acid, acetophenone, trans-3-Hexenedioic acid, and 9-octadecenoic acid (Z)-methyl ester.

methanolic extract showed a broad spectrum of very significant antibacterial activity of producing a clear zone of inhibition against *Streptococcus mutans*, *Streptococcus sanguis*, *Stephylococcus aureus* and *Lactobacillus acidophilus* and *Lactobacillus casei*.

50% methanolic extract at 5mg/ml and 2.5mg/ml concentration shows slightly higher zone of inhibition (ranging from 28 to 31 mm and 19 to 26 mm), and 10% methanolic 5mg/ml and 2.5mg/ml extract shows slightly small zone (ranging from 26 to 22 mm and 24 to 20 mm) Based on the preliminary screening assay, the *Murraya Koenigii* extracts were further evaluated to determine the minimum inhibitory concentration (MIC). MIC was determined as the lowest concentration of the extract, which inhibited the growth of the tested microorganisms. Results exhibit the profound and promising activity of *Murraya Koenigii* on BHI 0.090 mg/ml (**Table 3**).

These phytochemicals have been shown to possess antimicrobial, antioxidant, anti-cancer, hypercholesterolaemic and anti-ulcerogenic activities (**Table 4**).

TABLE 4: SHOWING IDENTIFIED COMPONENT IN THE METHANOLIC EXTRACT OF *MURRAYA KOENIGII* BY GC-MS.

Retention time	Name of Molecule	Molecular Formula	Molecular Weight	Active biological activity**
4.48	Propane, 1,1,3 triethoxy	C ₉ H ₂₄ O ₂	176	Used in the formulation of drugs by both oral and transdermal delivery routes
5.96	Acetophenone	C ₈ H ₈ O	120.058	Antibacterial, fungicide, pesticide, hypnotic, perfumery, sporofic
6.50	9-Heptadecanone	C ₁₇ H ₃₄ O	254	Not known
9.20	Oleic Acid	C ₁₉ H ₃₆ O ₂	296	5-Alpha-Reductase-Inhibitor, Allergenic, Alpha-Reductase-Inhibitor, Anemiagenic, Antiallopecic, Antiandrogenic, Antiinflammatory, Antileukotriene-D4 (Anti-platelet activating factor), Cancer Preventive, Choleric, Dermatitigenic Flavor, Hypocholesterolemic, Insectifuge Irritant, Percutaneostimulant, Perfumery, Propecic
10.59	9-Heneicosanone	C ₂₁ H ₄₂ O	310	Not known
12.12	Octadecanoic acid, methyl ester (Stearic acid methyl ester)	C ₁₉ H ₃₈ O ₂	298	Anti inflammatory, hypocholesterolemic cancer preventive, hepatoprotective, anti histaminic antieczemic, antiacne, 5-Alpha reductase inhibitor, anti androgenic, anti arthritic, anti coronary, insectifuge
16.11	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)	C ₂₄ H ₆₀ O ₆ Si ₆	612	Antidepression, Liver problems, panic disorders and diabetes
18.64	Linoleic acid	C ₁₉ H ₃₄ O ₂	294	Antioxidant,
18.12	ethyltrimethylsilyl ester	C ₈ H ₁₆ O ₄ Si	232.	Not known
22.69	Tetradecanoic acid, 12-methyl-, methyl ester	C ₁₆ H ₃₂ O ₂	256.24	Hypercholesterolemic
23.90	Hexadecanoic acid, (Palmitic acid) methyl ester	C ₁₇ H ₃₄ O ₂	270	Antioxidant, hypocholesterolemic nematocide, pesticide, anti androgenic flavor, hemolytic, 5- Alpha reductase inhibitor
25.92	cis-2-Hexen-1-ol, trimethylsilyl ether	C ₆ H ₁₂ O	172.128	Antioxidant, hypocholesterolemic nematocide, pesticide, anti-androgenic flavor, hemolytic, 5-Alpha reductase inhibitor

** Activity source: Dr. Duke's Phytochemical and Ethnobotanical Database

DISCUSSION: Oral cavity is the manifested for microorganism a broad group of microorganisms *Streptococcus mutans*, *Streptococcus sanguis*, *Staphylococcus aureus* and *Lactobacillus acidophilus* and *Lactobacillus casei* play a major role in dental caries formation⁵. Antibiotics (antimicrobials) are often prescribed for the adjunctive treatment of dental caries and large use of these antibiotics, antibiotics resistance capacity are increasing, natural remedies are the strongest tool for the treatment of this infection causative agents of dental caries and dental plaque, isolated pathogens were tested for

morphological and biochemical's and compared with ATCC cultures¹⁷.

The therapeutic benefits of secondary metabolites of plant origin have been researched in several recent studies¹⁸. The past decade has seen considerable changes in the opinion regarding the applications of ethnopharmacological therapeutics. In the present study, *Murraya Koenigii* was assessed for its antioxidant potential using DPPH, ABTS and FRAP assays. This is the first time that, this study has been carried out on human cariogenic pathogens.

Phytochemical analysis of *Murraya Koenigii* extracts revealed the presence of alkaloids, tannins, steroids, and saponins. All of these compounds have been shown to be potent antioxidants and antimicrobial¹⁹. Saponins and tannins are known to have analgesic and anti-inflammatory properties and have considerable cancer-prevention properties²⁰. Alkaloid-containing plants have been used by humans for centuries for therapeutic and recreational purposes. *Murraya Koenigii* containing these compounds may serve as a potential source of bioactive compounds in the prevention of dental caries.

In the DPPH assay, antioxidants could reduce the stable radical DPPH to the yellow-coloured DPPH. This suggests that the plant extracts contained compounds capable of donating hydrogen to a free radical to remove the extra electron (which is responsible for the activity of free radicals). Proton-radical scavenging is an important attribute of antioxidants²¹.

The protonated radical ABTS has characteristic absorbance maxima at 734 nm, which decreases with the scavenging of proton radicals. The scavenging activity of the ABTS radical by the plant extracts was found to be appreciable. This implies that the plant extracts may be useful for treating free radical-related pathological damage (especially at a higher concentration).

The FRAP assay measures the reducing ability of antioxidants against the oxidative effects of ROS. The reducing potentials of the methanolic extracts of the *Murraya Koenigii* were estimated from their ability to reduce the TPTZ–Fe(III) complex to the TPTZ–Fe(II) complex. Assays such as ABTS, FRAP and DPPH have shown that plant extracts may be useful for treating free radical-related cellular damage. In the present study, the extract of *Murraya Koenigii* were subjected to phytochemical evaluation and GC–MS analysis but the compounds responsible for the antioxidant and antimicrobial activity need to be explored²².

The present study has shown that *Murraya Koenigii* plant is potentially a rich source of antibacterial agents. *Murraya Koenigii* plant extracts tested, inhibited the growth of all pathogens and very effective as compared with standard antibiotic Amoxicillin.

The current study suggests that crude extracts demonstrating anti dental caries activity could result in the discovery of new chemical classes of antibiotics that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases. In addition, research is continuing to identify and purify the active compounds responsible for antioxidant and antimicrobial activity.

CONCLUSION: This study is a preliminary evaluation of antimicrobial and antioxidant activity of the *Murraya Koenigii* plants. It indicates that *Murraya Koenigii* plant have the potential to generate herbal metabolites. The crude extracts demonstrating anti dental caries activity could result in the discovery of new chemical classes of antibiotics that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases. The potential for developing antimicrobials from higher plants appears rewarding as it leads to the development of new drugs which is needed today.

Further research is necessary to find the active compounds within these plants with their full spectrum of efficacy. However, the present study of *in vitro* antibacterial activity of *Murraya Koenigii* forms primary platform for further phytochemical and pharmacological studies in human Cariogenic pathogens

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