



E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 1223-1228
Received: 10-05-2019
Accepted: 12-06-2019

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Evaluation of antimicrobial activity of ethanolic extract of *Murraya koenigii* against *S. mutans*

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Abstract

Periodontal disease is a bacterial infection that affects the gums and bone supporting the teeth. The present study was aimed to evaluate the Antimicrobial activity of *Murraya koenigii* extract against *Streptococcus mutans*. Ethanol extract was subjected to phytochemical screening, thin layer chromatography, total phenolic content, antioxidant activity, antimicrobial activity. Phytochemical analysis of the extract of *Murraya koenigii* indicated the presence of phytochemicals such as flavanoids, phenolics, alkaloids, and terpenoids. Thin layer chromatography was performed. Antioxidant assays were evaluated by assessing DPPH radical scavenging activity against standard butylated hydroxyanisole (BHA). From this study it can be concluded that *Murraya koenigii* shows significant antibacterial effect against gram positive *S mutans*. The *Murraya koenigii* were found to contain gallic acid and other phenolics which may be responsible for antibacterial activity. The studies will be explored for development of novel formulation using *Murraya koenigii* extract for periodontal diseases.

Keywords: *Murraya koenigii*, antimicrobial activity, *S mutans*, antioxidant activity, thin layer chromatography, total phenolic content

Introduction

Periodontal diseases are a group of inflammatory and localised infections caused by microbes affecting supporting tissue of the teeth, gingival, periodontal ligament and alveolar bone. Inflammation of gingiva is due to gingivitis whereas further extension of inflammation into deeper tissues accompanied with bone loss is termed as 'Periodontitis' [1]. It is a bacterially induced, localized, chronic inflammatory disease that destroys connective tissue and bones which supports the teeth [2]. It is one of the severe forms of gum disease and The *Murraya koenigii* were found to contain gallic acid and phenolics which may be responsible for antibacterial activity if untreated periodontal condition leads to spread of infection systematically, consequently found to be associated with cardiovascular diseases, atherosclerosis, overt nephropathy and end – stage renal disease. As per the estimates of National Examination Survey (NHANES) during 2009 and 2010, there is high prevalence of periodontitis among older people in United States (US). The survey reports more than 47% of the adults over the age of 30 years and 64% of the adults over the age of 65 years are suffering from either moderate or severe periodontitis. The prevalence of periodontitis was highest in men, Mexican American, adults having less than high school education, adults below poverty and current smokers [3].

First line strategy for periodontal disease treatment involves conventional mechanical therapy i.e. scaling and root planning (SRP) which can be improved by the overall gingival health and halts the progression of disease [4]. US patent US5676544 reports an instrument for subgingival SRP and maintenance of periodontal health. The instrument consist of probe which is used for the periodontal patient by inserting into the periodontal pocket present between the tooth and gum. Radiation therapy is also popular for killing pathogens residing inside the periodontal pocket [5]. US7090497 claims elimination of infectious microbes by selective irradiation with high- energy antiseptic laser pulses [6].

Murraya koenigii (Curry Leaves) commonly known as *curry leaf* or *kari patta* in Indian dialects which belongs to Family Rutaceae mainly contains more than 150 genera and 1600 species [7]. *Murraya Koenigii* (Curry Leaves) is staple in Indian dishes which is well known for flavour and used as condiment in the preparation of Curry powder, pickle, chutney, sausages, seasonings, oil and shampoo and these are available in the market. The branches of *M. Koenigii* are very popular for cleaning the teeth used as datum and it is also said that the branches of *M. Koenigii* are used to strengthen gums and teeth's [8, 9]. It is a popular ayurvedic home remedy used extensively in South India. Apart from this, curry leaves has a number of medicinal properties.

Curry leaves contain many important ingredients like carbohydrates, proteins, fibres, calcium, phosphorus, iron, magnesium, copper, minerals and vitamins like nicotinic acid, vitamin B, A, C and E, antioxidants, plant sterols and amino acids, glycosides and flavonoids [8]. *Murraya koenigii* leaves are recognized to be the good source of carbazole alkaloids. The other phytochemicals isolated and characterized from the Curry leaves are alkaloids such as mahanine, koenine, koenigine, koenidine, girinimbiol, girinimibine, koenimbine, O-methyl murrayamine A, O-methyl mahanine, isomahanine, bismahanine, bispyrayafoline, and rich source of iron [10]. *Murraya koenigii* possess anti inflammatory activity [11], anti – ulcer activity [12], antibacterial activity [13], antimicrobial activity [14], anti-helminthic activity [15], antioxidant activity [10].



Fig 1: *Murraya koenigii* leaves

Streptococcus mutans is a gram positive, non capsulated coccus, about 0.75 μ m in diameter when grown on a medium with a neutral or alkaline reaction. *Streptococcus Mutans* are a major pathogenic agent of dental caries. In the present investigation, an attempt has been made to investigate antimicrobial screening of leaf extracts of *Murraya koenigii* against pathogenic microorganisms [16].

Materials and methods

Materials

Ethanol, Sodium carbonate, folin reagent, DPPH, BHA, Concentrated Sulphuric acid, Chloroform all chemicals which are used are AR grade. All these were purchased from LOBA chem. Pvt Ltd. Mumbai.

Collection of Plant

Murraya koenigii was purchased from local market and was authenticated by a botanist in Botanical survey of India, Pune, Maharashtra. Specimen no is SAP 01 and the certificate no is BSI/WRC/IDEN.CER./2019/H3/127.

Physico-chemical characteristics of *Murraya koenigii* powder

Determination of Loss on drying

The loss on drying was determined by weighing 2 g of powder of *Murraya koenigii* in an evaporating dish and then dried in an oven at 105 °C till constant weight was obtained. The weight after drying was noted and loss on drying was calculated. The percentage was calculated on the basis of sample taken initially.

Determination of Total Ash

The total Ash value of powder of *Murraya koenigii* was determined by incinerating 1gm of accurately weighed crude powder in a tared silica crucible. It was incinerated in a muffle furnace at a temperature not exceeding 450 °C until free from carbon, then cooled and weighed.

Determination of Acid insoluble ash

The total ash obtained was boiled for 5 min with 25 ml dilute HCL. The insoluble matter was collected on the filter paper placed in a Gooch crucible, washed with water and heated till the constant weight was obtained. The percentage of acid insoluble ash was calculated with reference to the sample taken initially.

Determination of Water soluble Ash

The Water soluble Ash was determined by boiling the obtained ash for 5 minutes with 25 ml of water, insoluble matter was collected in a Gooch crucible on ashless filter paper, washed with hot water, ignited for 15 minutes at a temperature not exceeding 450 °C. Weight of the insoluble matter from the weight of ash was subtracted. The difference in weight represents the water – soluble ash. The percentage of Water soluble ash was calculated with reference to the sample taken initially.

Determination of Water soluble extractive

Water soluble extractive value was determined by macerating 5 g of *Murraya koenigii* powder with 100ml of water the specified strength in a closed flask for 24 hrs, shaken frequently for 6 hrs and allowed to stand for 18 hrs. Filtered and evaporated 25 ml of the filtrate to dryness in a dish and was dried at 105 °C till constant weight and weighed. The percentage of water soluble extractive was calculated with reference to air dried *Murraya koenigii* powder.

Determination of Alcohol Soluble Extractive

Alcohol soluble extractive value was determined by macerating 5 g of *Murraya koenigii* powder with 100ml of alcohol the specified strength in a closed flask for 24 hrs, shaken frequently for 6 hrs and allowed to stand for 18 hrs. Filtered and evaporated 25 ml of the filtrate to dryness in a dish and was dried at 105 °C till constant weight and weighed. The percentage of water soluble extractive was calculated with reference to air dried *Murraya koenigii* powder [17].

Preparation of Extract

250 grams of powdered leaves of *Murraya koenigii* were extracted using 250 ml of solvent like ethanol in soxhlet apparatus separately for 24 hours and they were concentrated by evaporation process. The cold extract thus obtained and were filtered by whatmann No. 1 filter paper into a conical flask and were allowed to dry at room temperature at normal atmospheric pressure. The obtained crude extract were stored in closed container and used for preliminary qualitative Phytochemical analysis [18].



Fig 2: Extraction by soxhlet Apparatus

Extractive value = weight of plant extract/weight of dry powdered Sample \times 100

Characterization of *Murraya koenigii* Extract

Determination of Solubility

The qualitative solubility of *Murraya koenigii* was determined in different solvents such as Ethanol, methanol, chloroform, ethyl acetate, diethyl ether, benzene, n – octyl alcohol, isopropyl alcohol, n – hexane, polyethylene glycol 400, toluene, acetone and formic acid [18].

Phytochemical Screening

The extract was subjected to preliminary phytochemical screening for the detection of various plant constituents and there results are in table no. 1 [19].

Thin layer Chromatography

Preparation of plate

The adsorbent used for the preparation of thin layer plate (TLC) as a stationary phase was silica gel G. The absorbent silica gel coated on an Tlc plate of 7.3 cm in length, 2.5 cm breadth and 0.3 cm thick in length. The extract (*Murraya koenigii* leaves) was taken n dissolved in the ethanol for the spotting purpose. Small spot of the solution containing sample i.e. *Murraya koenigii* leaves extract was applied on the plate 1.0 cm from the bottom mark.

Spotting and Development, Visualization and Detection

The sample was spotted on the TLC plate and was allowed to dry before the plate was placed into the chromatographic chamber which is completely saturated with a mobile phase. The mobile phase used solvent 10ml as Chloroform. The spot was detected by viewing under the UV at 254 and 366 i.e short and long wavelength [18].

Total Phenolic Content by Gallic acid

Total soluble phenolic in the ethanolic extract of *Murraya koenigii* were determined with Folin – ciocalteu reagent according to the standard method using BHA as a standard. Briefly 0.1 ml of extract solution (contains 1000 µg extract) in a volumetric flask diluted ethanol (46 ml). About 1ml of Folin – Ciocalteu reagent was added and mixed thoroughly. After 3 minutes 3ml of Sodium carbonate (2%) was added, then the mixture was allowed to stand for 2 hours with intermittent shaking. The absorbance was measured at 760 nm [20]. The concentration of total phenolic compounds in the *Murraya koenigii* determined as microgram of BHA equivalent by using an equation as given below:

$$\text{Absorbance} = 0.001 \times \text{BHA } (\mu\text{g}) + 0.0033$$

Antioxidant activity

Free radical scavenging activity by DPPH method

Free radical scavenging activity of ethanol extract of *Murraya koenigii* were measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging method. Further, 0.1 mM Solution of DPPH was prepared in methanol. Different concentrations of test drug solution (50 – 100 µg/ml) were prepared. To this 5 ml of methanolic solution were added, shaken well and mixture was kept at room temperature for 30 min. Then the absorbance was measured at 517nm by using a spectrophotometer (UV– VIS Shimadzu, Model-1800). Reference standard compound used was BHA (10-60 µg/ml). The IC₅₀ Value is the concentration of sample required to inhibit 50% of the DPPH free radical. The IC₅₀ for the sample was calculated using log dose inhibition curve. Thus, the Lower absorbance of the reaction mixture indicates higher

free radical activity. The percent DPPH scavenging effect was calculated using the following equation [21].

$$\text{DPPH scavenging effect (\%)} = 100 \times A1/ A0$$

Where,

A0 = Absorbance of the control reaction

A1 = Absorbance of the standard sample or extract

Antimicrobial Activity

Minimum Inhibitory Concentration of *Murraya koenigii* extract

Bacterial Strains

Test bacteria used were *Streptococcus mutans* (MTCC 890) and this microorganism were obtained from IMTECH Chandigarh.

Determination of Minimum Inhibitory Concentration

Broth dilution method was used to measure the MIC values. The *Murraya koenigii* extract was dissolved in the respective solvent to prepare an antibiotic solution containing 10mg/ml. The reconstituted extract was serially diluted 2 fold in Mueller Hinton broth to obtain various concentrations of the stock, 10,5,2.5, 1.25, 0.625, 0.312, 0.156, 0.0078, 0.039, 0.0195 mg/ml and were assayed against the test organisms. The tubes were incubated at 37 °C for 24 hrs. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth [22].

Result and discussion

The physico-chemical characterization of *Murraya koenigii* powder as shown in Table no.1. The preliminary phytochemical screening revealed the presence of alkaloids, carbohydrates, cardiac glycosides, flavonoids, phenols, phylobatannins, tannins, terpenoids and quinones in the ethanol extract of *Murraya koenigii* as shown in the Table no.2. In this study evaluated the antibacterial effect of the crude extract obtained from the leaves of *Murraya koenigii* using ethanol as a solvent against *streptococcus mutans* on the basis of minimum inhibitory concentration (Table no. 2) by following the broth dilution method [23].

Physico-chemical characterisation

Table 1: Physico-chemical characteristics of *Murraya koenigii* powder

S. No	Particulars	Result
1.	pH	6.3-6.4
2.	Loss on Drying	0.15%
3.	Total Ash	36.6%
4.	Acid insoluble Ash	1.775%
5.	Water soluble Ash	6.205%
5.	Water soluble extractive	89.5%
6.	Alcohol soluble extractive	74%

Physico-chemical characteristics of *Murraya koenigii* Extract

The extract of *Murraya koenigii* leaves was found to be dark greenish, aromatic, bitter in taste [24]. *Murraya koenigii* extract was found to be soluble in Ethanol, methanol, chloroform, ethyl acetate, diethyl ether, benzene, n – octyl alcohol, isopropyl alcohol, n – hexane, polyethylene glycol 400 and insoluble in in Acetone, toluene and formic acid [24, 17].

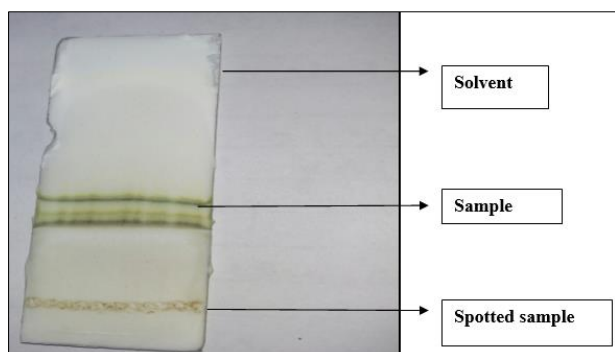
Phytochemical Screening

Table 2: Phytochemical screening of *Murraya koenigii* extract.

S. No	Phytochemicals	Test	Observation	Inference
1.	Alkaloids	Wagner's test	Red brown precipitate	Alkaloids Present
		Mayers test	Creamy ppt	Alkaloids present
2.	Carbohydrates	Molisch's test	Violet ring	Carbohydrate present
		Fehlings test	Brick red ppt	Carbohydrate present
3.	Cardiac glycosides	Keller killiani test	Brown/violet/green ring	Cardiac glycosides present
4.	Flavonoids	Shinoda's test	Red colour	Flavonoids present
5.	Phenols	Ferric chloride test	Deep blue colour	Phenols present
6.	Phylobatannins	Precipitate test	Red ppt	Phylobatannins present
7.	Amino acids & Proteins	Ninhydrin test	-ve	Amino acids & proteins absent
8.	Tannins	Ferric chloride solution test	Green colour	Tannins present
9.	Terpenoids	Sakowski test	Reddish brown ppt	Terpenoids present
10.	Quinones	Concentrated Hcl + extract	Yellow ppt	Quinones absent

Thin layer Chromatography

The TLC plate was observed in the UV cabinet at 254 and 366 i.e. short and long wavelength. The Rf Value was found to be 0.46 and thus it reveals the presence of Flavonoids. The *Murraya koenigii* leaves extract contains flavonoids. Flavonoids are well known as antibacterial agents against a wide range of pathogenic microorganisms. It is reported that the flavonoids shows their antibacterial activity against the *S. mutans* [25].

Fig 5: Thin layer Chromatography of *Murraya koenigii* Extract

Total Phenolic Content

Total phenolic content of ethanolic leaf extract of *Murraya koenigii* was estimated by Folin-Ciocalteu reagent and results were expressed as equivalents of Gallic acid. The phenolics are the well known secondary metabolites and comprise a large group of biologically active compounds. Total polyphenols in the ethanolic extract sample was expressed in terms of Gallic acid equivalents (GAE) with the standard curve equation of $y = 0.003x - 0.005$ ($r^2 = 0.996$) [10]. Hence, the total phenolic content was found to be 1.35%.

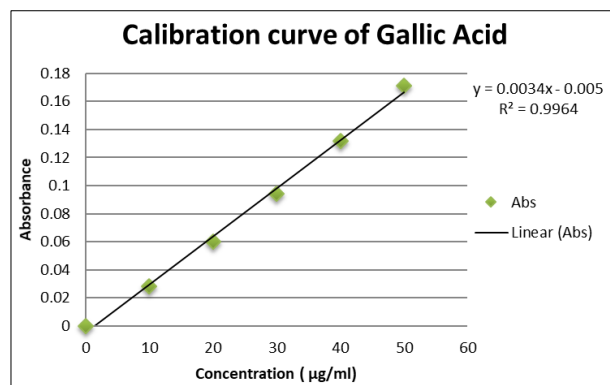
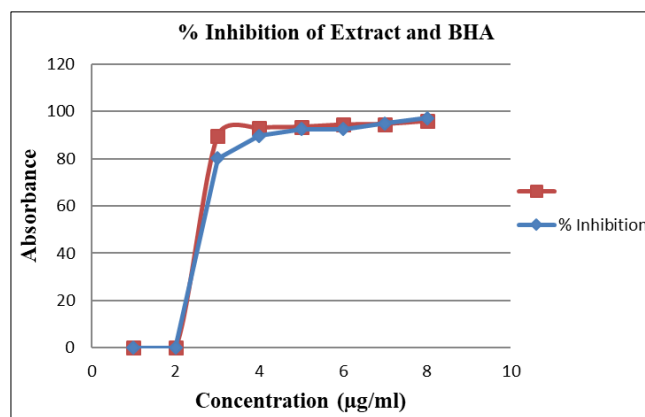


Fig 3: Calibration Curve of Gallic Acid

Antioxidant Activity

Extract of *Murraya koenigii* posses Antioxidant activity. Decolouration due to reaction of antioxidant in sample with the suitable free DPPH radical was measured spectrophotometrically. Reduction of DPPH can be observed by the decrease in the absorbance at 517 nm. In this study, the antioxidant activity of *Murraya koenigii* extract was performed by comparing standard solution Butylated Hydroxyanisole (BHA). The *Murraya koenigii* extract shows minimum percent Inhibition as compared to standard i.e. BHA [10, 13]. As the concentration increases the percent inhibition also increases. The IC₅₀ value of *Murraya koenigii* shows higher value as compared to the standard. The IC₅₀ value of *Murraya koenigii* and BHA was found to be 4.10 µg/ml and 1.02 µg/ml.

Fig 4: % Inhibition of *Murraya koenigii* Extract and BHA

Hence, the Antioxidant activity of *Murraya koenigii* and BHA was found to be 79.80% and 97.04% respectively.

Antimicrobial activity

The antimicrobial activity of the extract of *Murraya koenigii* leaves was quantitatively assessed on the basis of Minimum Inhibitory Concentration by Broth dilution method. The test organisms were inoculated with *Murraya koenigii* leaf extract at 37 °C for 24 hrs to evaluate antimicrobial activities. The phenolic content of *Murraya koenigii* leaf extract was found to be 1.35% which may be responsible for its promising antimicrobial activity. It is reported that the gallic acid shows inhibitory activity against the *S mutans* [26]. The *Murraya koenigii* in particular ethanolic extract and others exerted significant antibacterial activity against gram positive *S mutans*. The *Murraya koenigii* were found to contain gallic

acid and other phenolics which may be responsible for antibacterial activity. Similar findings were noted in literature [13, 27, 28]

Table 2: *Murraya koenigii* Extract effect on *Streptococcus mutans*

Bacteria	Media	Mic (mg/ml)
<i>Streptococcus mutans</i>	Mueller Hinton	0.625 mg/ml

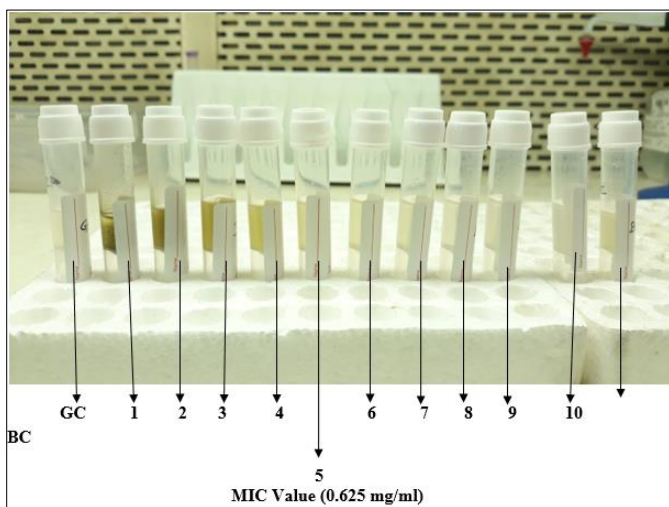


Fig 6: MIC Value of *Murraya koenigii* extract in *S. Mutans* after Incubation

GC– Growth Control (*Streptococcus mutans* positive growth)
1-1 mg/ml, 2- 5 mg/ml, 3 – 2.5 mg/ml, 4 – 1.24 mg/ml, 5 – 0.625 mg/ml,

6 – 0.312mg/ml, 7 – 0.156 mg/ml, 8 – 0.078 mg/ml, 9- 0.039 mg/ml, 10 – 0.0195 mg/ml

BC – Broth Control

The Minimum growth was observed in the Test tube no. 5 which having the concentration 0.625 mg/ml.

Conclusion

The present study revealed that the ethanol extract from the leaves of *Murraya koenigii* exhibited Antimicrobial properties which explains the basis for its use in traditional medicines to treat dental caries. Ethanol extract showed significant inhibitory activity against streptococcus mutans. The present study concludes that the *Murraya koenigii* may serve as a potential source of bioactive compounds in the prevention of dental caries. The *Murraya koenigii* were found to contain gallic acid and phenolics which may be responsible for antibacterial activity. The potential for developing antimicrobials from higher plants appears rewarding as it leads to the development of new drugs which is required today. The studies will be explored for development of novel formulation using *Murraya koenigii* extract for periodontal diseases.

Acknowledgement

The Authors are greatly thankful to the JSPM's Jayawantrao sawant college of Pharmacy and research for providing Technical assistance.

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