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Antimicrobial Activity of *Melia azedarach* Against *Porphyromonas gingivalis*, Acute Oral Toxicity Characterization and Development of DNA Minibarcode Based Markers for Identification from Allied Plants

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Key words
***Melia azaderach, Azadirachta indica, Porphyromonas gingivalis*, Antimicrobial, Real-time PCR**

ABSTRACT

Porphyromonas gingivalis is one of the keystone bacteria which causes periodontitis in oral cavity and has ability to infect many peripheral organs. Its infection is also associated with Alzheimer's disease, respiratory infection, diabetes and cardiovascular disease. Generally, local antiseptics or antibiotics are used to treat the periodontal diseases which has certain limitations like tooth staining, irritation in oral cavity, altered taste and mainly progressive antibiotic resistance in pathogens. To overwhelm the limitations of synthetic drugs, research has been focused to search new natural herbs or medicinal plants for treatment of oral ailments. The aim of the present study was to explore the potential and safety of *Melia azedarach* leaf extracts against *P. gingivalis* in comparison to the allied/very similar plant species *Azadaracta indica* extracts as natural substitute of chemical agents or antibiotics. The results revealed that among different extracts of *Melia azedarach*, ethanol extract was most potent against *P. gingivalis* with MIC value 156 µg/ml with effectivity half to the ethanolic extract of *Azadaracta indica* (MIC 78 µg/ml). *In vivo* toxicological studies in mice showed that safety profile of ethanolic extract of *Melia azaderach* leaves was higher than *Azadaracta indica* by oral route of administration. *Melia azedarach* and *Azadirachta indica* are sold under same vernacular name in IndoPak but differ from each other in therapeutic efficiency and safety profile therefore for quality assurance and product standardization, minibarcodes were developed for identification by single step PCR. The mechanism of action of *Melia azedarach* ethanolic extract against *P. gingivalis* was also explored. Therefore, *Melia azedarach* can also be considered as potential and safe candidate as natural therapeutic against *P. gingivalis*. However, further experiments should be performed to evaluate the cytotoxicity and genotoxicity for prolong use.

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INTRODUCTION

Periodontal disease is one of the most leading common health problems in the human communities (Sheiham, 2005). Dental plaque, a biofilm of microorganisms located at the interface of teeth and the gingiva comprised of many bacterial species, is one of the primary factors in the development of caries and periodontal disease (Newman *et al*., 2006). Dental plaque can cause gingivitis (gingival

inflammation, most common forms of periodontal disease) that further leads to the periodontitis (Bansal *et al*., 2012). In gingival health, plaques are typically encompassed of Gram-positive facultative aerobic bacteria but in case of gingivitis and periodontitis, Gram-negative bacteria are present in addition. The potential well-recognized bacteria involved in disease progression are *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* or *Treponema denticola*, as well as *Aggregatibacter actinomycetemcomitans* (Teles *et al*., 2013). Among these *P. gingivalis* has ability to escape. Its infection is also associated with Alzheimer's disease, respiratory infection, diabetes and cardiovascular disease and preterm birth (Singhrao *et al*., 2015; Belstrom *et al*., 2011; Kim and Amar 2008; Hashioka *et al*., 2019; Dominy *et al*., 2019).

Treatment of periodontal diseases mainly rely on removal of dental plaque either mechanically by maintaining oral hygiene such as by tooth brushing or by chemical plaque control using local antiseptic (mouthwash) or antibiotics (Sanz *et al*., 2012). A broad-spectrum antiseptic, chlorhexidine (CHX) is generally used for chemical dental plaque control (Sreenivasan and Gittins, 2004) but prolong use of CHX mouthwashes can cause irritation of oral mucosa, teeth staining, altered taste sensation and increased dental calculus formation (James *et al*., 2017). In clinical practice, a variety of antibiotics such as amoxicillin, fluoroquinolones, metronidazole, tetracycline, clindamycin, and azithromycin are employed for the treatment of periodontitis (Slots, 2002; Beikler *et al*., 2004). However, the widespread use of antibiotics has several negative effects, such as resistances in bacteria, alterations of the gut microbiota and sometimes direct renal and hepatic damage (Rams *et al*., 2014). All these facts demand the search and development of novel antimicrobial agents for the treatment of periodontal diseases.

Plants and their extracts have been used for therapeutic purpose and generally considered as safe, effective and economical. Owing antibacterial and anti-inflammatory activity, herbal medicines (as mouthwash) have been used in dentistry to inhibit microbial growth, reduce inflammation, soothe irritation, and relieve pain with remarkable control of plaque and gingivitis has been reported (Taheri *et al*., 2011; Rodrigues *et al*., 2013). Commonly used herbs for periodontal diseases includes *Acacia nilotica* (Sahni *et al*., 2016), *Aloe barbedensis* (Kalra *et al*., 2017), *Salvadora persica* (Niazi *et al*., 2016), *Eugenia caprophylatta* (Nuñez and D'Aquino 2012), *Nigella sativa* (Al-Attas *et al*., 2016), *Azadirachta indica* (Lakshmi *et al*., 2015), *Propolis resin* (Bazvand *et al*., 2014) and *Syzygium aromaticum* (Chaieb *et al*., 2007).

A. indica (from Meliaceae family) twigs are well known for their ethnomedicinal uses in mouthwash and toothpaste as oral deodorant to relieve toothache, to reduce gum inflammation and for cleaning of teeth. It is very effective against periodontal pathogens causing dental caries and plaque (Chava *et al*., 2012; Packia *et al*., 2012; Elavarasu *et al*., 2012). Antibacterial activity of *A. indica* ethanol extract has been recently reported against *P. gingivali* with MIC 64 µg/ml (Carrol *et al*., 2020). *M. azedarach*, a plant from meliaceace family is very similar to the *A. indica*. It has ethnomedicinal use for different ailments and against periodontal diseases as well but differs in therapeutic properties from *A. indica* (Khan *et al*., 2011; Khalid *et al*., 2017; Singh *et al*., 2020; Lee and Kim, 2019). The pharmacological activity of *M. azedarach* has not been reported against *P. gingivali* yet.

Therefore, the aim of the present study was to evaluate the activity of *M. azedarach* extracts against *P. gingivali* and *in vivo* toxicity in mice comparative to the *A. indica*. For quality assurance, DNA markers based on minibarcodes were also developed to identify the both plant species. The underlying mechanism of *M. azedarach* ethanolic extract against *P. gangivalis* was also explored.

MATERIALS AND METHODS

Plant material and extract preparation

The leaves of *Melia azedarach* and *A. indica* were collected from Lahore city. The samples were verified by Dr. Zahoor Ahmed Sajid, Department of Botany, University of the Punjab, Lahore, Pakistan and voucher specimen was also deposited in herbarium (voucher number IBB-79/PU) in School of Biochemistry and Biotechnology, University of the Punjab, Lahore, Pakistan. The leaves were washed to remove the dust and dried at room temperature. Then crushed to fine powder and used for preparation of crude extract using various solvents including water, ethanol methanol, ether, n-hexane. 10 g of powder was mixed with 100ml of solvent and kept for 24 h at room temperature with continuous agitation. The filtrate was collected by centrifugation at 8000 rpm for 5 min then concentrated in rotary evaporated at 50 °C. The dried extraction products were weighed, dissolved in dimethyl sulfoxide (DMSO) to the concentration of 100 mg/ml and stored at -20 °C under dark conditions till further use.

Bacterial strain and growth conditions

The stock culture of bacterial strain *P. gingivalis* was obtained from Dentistry Department of Akhtar Saeed Medical Hospital, Lahore, Pakistan. The strain was grown in brain heart infusion (BHI) medium supplemented with 1 µg/mL menadione and 5 µg/mL hemin and blood agar plates supplemented with 5 % defibrinated sheep's blood, 1 µg/mL menadione and 5 µg/mL hemin at 37 °C under

anaerobic conditions with 80% nitrogen, 10% hydrogen and 5% CO_2. Black and mucoid colonies of *Porphyromonas gingivalis* were obtained after 48-72 h of growth.

Evaluation of anti- P. gingivalis *activity*

The screening of crude extracts for anti- *P. gingivalis* activity was performed by disc diffusion method. Briefly, the supplemented blood agar plates were seeded with 100 µl of fresh culture of *P. gingivalis* containing 1x 10^6 CFU/ml. After inoculation, plates were placed at 37 °C for 15 min. The sterile disc of 6 mm (millimeter) prepared from whatman filter paper impregnated with different concentration of each extract were placed separately on plates seeded with *P. gingivalis* culture and incubated at 37 °C for 48 h under anaerobic conditions. The zone of inhibition was measured. The experiment was performed thrice and the mean of readings is mentioned in the results. Disc impregnated in 0.12 % chlorhexidine and DMSO were used as positive and negative control respectively. The inter and intra group comparison were done using student's t-test.

Determination of minimum inhibitory concentration (MIC)

The method of Minami *et al*. (2019) was used to determine the MIC of extracts against *P. gingivalis*. Briefly, 200 µl culture of *P. gingivalis* in supplemented BHI medium with 1x 10^6 CFU/ml was added in each well of 96 well flat bottom non- tissue culture treated plate. The extracts were tested at different concentrations of two fold serial dilutions ranging from 10.0 mg/ml to 1.0 µg/ml. DMSO was used as vehicle control. The plate was incubated at 37 °C for 72 h under anaerobic conditions. The minimum concentration at which 90 % growth inhibition was recorded in comparison to vehicle control considered as MIC. Inhibition in growth was recorded by change in optical density at 600nm (OD_{600}) from start point to the end of incubation time (72 h). The experiment was performed in triplicate. The mean of readings and standard error was calculated using Microsoft Excel.

Growth kinetic assay

The rate of anti-*P. gingivalis* activity of extracts was determined using 1 x MIC of ethanol extract. The 48 h grown culture of *P. gingivalis* was adjusted to the 1x 10^6 CFU/ml with supplemented BHI media and 200 µl of culture was added per well in 96 well flat bottom non-tissue culture treated plate with 1 x MIC of extract. The culture was incubated at 37 °C for 72 h under anaerobic conditions. The culture aliquots were collected with interval of 4.0 h till the end of incubation period, diluted and plated on supplemented blood agar plates at 37 °C for 48 h under anaerobic conditions. The colonies were counted and results were illustrated as $\log_{10}$ CFU and plotted versus time.

Toxicological studies

The toxicological assessment of potent extracts was conducted in male albino mice with average weight of 50 g. Prior experiment, the mice were kept in animal house of School of Biochemistry and Biotechnology, University of the Punjab, Lahore for two weeks to acclimatize the laboratory environment at constant temperature of 25 °C with 12/12 h light/dark cycle. The extracts were administered orally according to the OECD (Organization for Economic Cooperation and Development) Guideline 423. The mice were divided in eight groups (ten mice per group) for each plant extract. Seven groups were given plant extract ranging from 100mg/kg to 6400 mg/kg and one group was given equal volume (1.0 ml) of carrier solvent (DMSO) for the period of 3.0 weeks. The LD_{50} of the extract was calculated using the arithmetic method of Karber as modified by Aliu and Nwude (1982) using the following equation.

$$LD50 = LD_{100} - \Sigma (Dd \times mm)/N$$

Where, LD_{50} median lethal dose; LD_{100}-dose which kill all the animal in a group; a, Dose difference; b, mean mortality; N, no. of animals in a group.

The acute toxicity of extracts was evaluated immediately after extract administration then after every two h during the day time. Any sign of toxicity such as: oedema, rising of fur, vomiting, increased respiration, sedation, change in skin color, eye color was recorded. The hematological and biochemical evaluation was also performed after the period of 3.0 weeks.

Molecular identification by development of minibarcodes based markers

For molecular identification of each plant, minibarcode based markers were developed for both *M. azedarach* and *A. indica*. The leaves of each plant were collected from five different locations of province Punjab, Pakistan. The leaves were washed thoroughly and stored at -80 °C. The DNA was isolated using GeneJet Genomic DNA Purification Kit (Thermo Scientific). The DNA barcode trnH-psbA was amplified from each plant species using commonly used standard plant trnH-psbA barcode primers (trnHf_05 5' CGCGCATGGTGGATTCACAATCC 3' and psbA3_R 5' GTTATGCATGAACGTAATGCTC 3') under conditions: 94 °C-30 sec, 56°C-30 sec, 72 °C-45 sec using PCR reaction mixture (50 µl) having 100 ng of DNA, 0.2 mm dNTPs, 1x Taq DNA polymerase buffer, 0.5 µM of each forward and reverse primer, 2.0 mM $MgCl_2$ and 2 units of Taq DNA polymerase. The amplicon was sequenced commercially and the sequence of each plant

species was compared using Clustal W software. Markers were developed to amplify the unique minibarcodes for each plant species (Table I) and also verified by PCR.

Table I. Primers of real-time PCR (He et al., 2020).

	Primer sequence (5'-3')
16S rRNA (control)	F= TGTAGATGACTGATGGTGAAA
	R= ACTGTTAGCAACTACCGATGT
Ferritin (*ftn*)	F= CGGCGAGGTGAAGATAGAAG
	R= CTCCTGAGAGAGACGGATCG
Hemolysin (*hm*)	F= ACGAAGCCTTGTTCTCCTCA
	R= CAATGAATATGCCGGTTTCC
Lysine-specifc cysteine protein-ase (*kgp*)	F= AGGAACGACAAACGCCTCTA
	R= GTCACCAACCAAAGCCAAGA
Hemagglutinin protein (*HagA*)	F= TAAATAAGGGCGGAGCAAGA
	R= GACGGAAAGCAACATACTTCG
Hemagglutinin protein (*HagB*)	F= TGTCGCACGGCAAATATCGCTAAAC
	R= CTGGCTGTCCTCGTCGAAAGCATAC

Gene expression analysis

The total RNA was extracted from *P. gingivalis* cells treated with *M. azedarach* ethanol extract at 1x MIC for 12 h using Trizol reagent (Invitrogen) according to the instructions of manufacturers and quantified by Nanodrop. The cDNA was synthesized from 1 µg of RNA using cDNA Revert-Aid First strand, cDNA synthesis Kit (Thermo Scientific) following the instructions of manufacturers. The expression of virulence factor genes (Table I) was quantified by real-time system in 20 µl reaction mixture prepared by using 2x maxima SYBR Green qPCR master mix (Thermo Scientific), 1 µl of each 10 µM qPCR primers (forward and reverse), 2 µl of 1: 5 folds diluted cDNA under the conditions: 95 °C for 10 sec, 60 °C for 20 sec and 72 °C for 15 sec. The gene expression was normalized against 16S rRNA expression. The gene expression levels were determined by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The experiment was performed in triplicate.

RESULTS AND DISCUSSION

Antimicrobial assay

In the present study, we evaluated the antimicrobial activity of *M. azedarach* comparative to the *A. indica* against *Porphyromonas gingivalis*. The extracts of *M. azedarach* prepared in different solvents were screened by disc diffusion method to identify the most effective extract against *Porphyromonas gingivalis*. Among the different extracts of *M. azedarach*, at the concentration of 10 mg/ml, ethanol extract was found most effective with zone of inhibition of 25 mm followed by methanol extract (17 mm) and aqueous extract (12 mm). The negligible antimicrobial activity was recorded of acetone, ether and n-hexane extracts against *P. gingivalis* even at the concentration of 100 mg/ml. In comparison to the *A. indica* extracts, *M. azedarach* extracts showed approximately 50 % less anti- *P. gingivalis activity* at the same concentrations. The extracts of both *A. indica* and *M. azedarach* showed anti-*P. gingivalis* activity in same pattern such as most effective extract was ethanol extract followed by methanol extract and aqueous extract (Fig. 1). The ethanol extract of *A. indica* at the concentration of 10 mg/ml showed same anti-*P. gingivalis* activity with zone of inhibition of 44 mm as of 0.12 % chlorhexidine (45 mm zone of inhibition). Saquib *et al*. (2019) reported the anti-*P. gingivalis* activity of ethanol extract of *S. persica* (Miswak) and *C. zeylanicum* (Ceylon cinnamon) with zone of inhibition of 15 mm for *S. persica* (Miswak) at the concentration of 6.25 ± 1.25 mg/ml and 18 mm for *C. zeylanicum* (Ceylon cinnamon) at 3.12±0.65 mg/ml (Saquib *et al*., 2019). As ethanol, methanol and aqueous extracts showed more promising results against *P. gingivalis*, therefore, used further for MIC assessment.

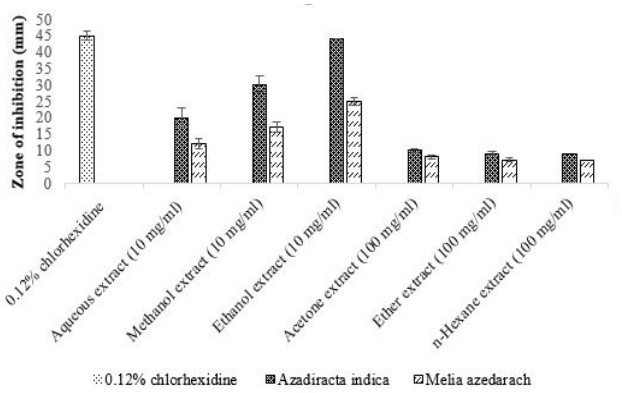

Fig. 1. Anti-*P. gingivalis* activity of different extract of *A. indica* and *M. azedarach*. Values are mean of three readings. Error bars are showing the standard error of three values.

Minimum inhibitory concentration

The MIC of two fold serially diluted extracts was determined. From the different extracts, ethanol extract of *A. indica* exhibited least MIC value of 78 µg/ml while of *M. azedarach* had MIC value of 156 µg/ml. For both plants, methanol extracts exhibited higher MIC values than ethanol extracts i.e., 312 µg/ml for *A. indica* and 625 µg/ml for *M. azedarach*. The aqueous extract of *M. azedarach* had highest MIC value of 5.0 mg/ml. The results of MIC

showed that *P. gingivalis* is susceptible to the *M. azedarach* but effectiveness is approximately half of the *A. indica*. The ethanol extract of *M. azedarach* was found most potent against *P. gingivalis* as of *A. indica* (Table II). Carrol *et al.* (2020) reported 64 µg/ml MIC of *A. indica* ethanol extract for *P. gingivalis* which is less than MIC value (78 µg/ml) of *A. indica* ethanol extract in the present study that may be due to the different geographical origin of *A. indica*. Although, *M. azedarach* showed higher MIC values than *A. indica* but it has less MIC values than *S. persica* (6.25 mg/ml) and *C. zeylanicum* (3.12 mg/ml) (Saquib *et al.*, 2019). Moheildin *et al.* (2017) screened 24 Sudanes plants for antibacterial activity against *P. gingivalis* activity. Most of the plants extracts had MIC ≤ 4.0 mg/ml while methanol extract of *Terminalia laxiflora* was found most potent with MIC value of 0.25 mg/ml. Whereas, MIC values of 50 % ethanol extracts of studied plants were higher than methanol extract with least MIC value of 0.5 mg/ml of *T. brownii* Fresen. Wang *et al.* (2018) reported MIC value 6.25 mg/ml of cinnamon (*Cinnamomum zeylanicum*) bark essential oil against *P. gingivalis*. Pandurain A, an active ingredient of *Kaempferia pandurata*, exhibited high antibacterial activity for *P. gingivalis* with MIC of 4 µg/ml (Park *et al.*, 2005). In the present study, the positive control chlorohexidine exhibited MIC value 0.58 µg/ml which is quite low value than plant extracts but prolong usage of CHX is associated with genotoxicity and cytotoxicity (Li *et al.*, 2014; Ribeiro *et al.*, 2004; Khan *et al.*, 2016).

Table II. Minimum inhibitory concentration of *A. indica* and *M. azedarach* extracts.

Extract	*Azadiracta indica*	*Melia azedarach*
Aqueous extract	1.25mg/ml	5.0 mg/ml
Methanol extract	312 µg/ml	625 µg/ml
Ethanol extract	78 µg/ml	156 µg/ml

Values are mean of three independent experiments.

Time kill kinetic assay

The real time effect of ethanol extracts on the growth of bacteria was investigated at 1 x MIC exposure (Fig. 2). The results revealed the decrease in the growth of *P. gingivalis* with increase in exposure time to extracts. There was no significant difference in growth inhibition effect of *A. indica* and *M. azedarach* at different exposure intervals. The growth was reduced to half after 12 h of exposure and complete inhibition in growth was observed after 24 h. The control 0.12 % CHX inhibited the growth of *P. gingivalis* after 12 h of exposure. The results of the study advocate the potent antibacterial activity of *M. azedarach* against *P. gingivalis*.

Median lethal dose for mice

Toxicological studies help in assessing the initial mode of toxicity and dose determination of drug (Akhila *et al.*, 2007). In the present study, ethanol extract was administered orally. The dose that resulted in 100 % mortality (LD_{100}) was 6400mg/kg and 12800mg/kg for *A. indica* and *M. azedarach*, respectively. Whereas, the median lethal dose (LD_{50}) was 5400mg/kg for *A. indica* and 6720mg/kg for *M. azedarach*. No morbidity was observed upto the 800 mg/kg of *A. indica* and 1600 mg/kg of *M. azedarach* in mice (Table III).

Table III. Determination of LD_{50} of ethanol extract of *Azadiracta indica* and *Melia azedarach* in mice (N=10).

Groups	*A. indica*				*M. azedarach*		
	Dose (mg/kg)	No. of animal dead	Mean mortality	Probity	No. of animal dead	Mean mortality (b)	Probity (a_*b)
A (control)	0	0	0	0	0	0	0
B	200	0	0	0	0	0	0
C	400	0	0	0	0	0	0
D	800	0	0	0	0	0	0
E	1600	1	0.5	400	0	0	0
F	3200	3	2	3200	2	1	1600
G	6400	7	5	16000	5	3.5	11200
H	12800	10	8.5	54400	10	7.5	48000

In the present study, test dose up to 12800 mg/kg have been used and 5400 mg/kg was found as median lethal dose. In different acute toxicological studies of *A. indica* using ethanol extract of leaves by oral administration, LD_{50} has been reported >5000 mg/kg in mice. Doses above 5000 mg/kg had not been used in the studies (Ghatule *et al.*, 2012; Achi *et al.*, 2018; Osenia and Akwetey, 2012). LD_{50} value > 5000 mg/kg is generally considered as safe. Therefore, ethanol extract from leaves of *A. indica* and *M. azedarach* is safe to use orally. The toxicity varies with the part of plant used for extract preparation and route of administration. As, intraperitoneal administration of leaves extract is highly toxic with LD_{50} of 31.62 mg/kg in mice. The methanol extract from stem bark of *A. indica* has moderate toxicity with 489.90 mg/kg LD_{50} in mice with oral route of administration while methanol extract from flower is safe orally with LD_{50} value of >12g/kg. Biu *et al.* (2010) reported the 4800mg/kg LD_{50} of *A. indica* leaf aqueous extract administered intraperitoneally in chicken (*Gallus gallus domesticus*). Regarding *M. azedarach*, no morbidity in mice up to 610mg/kg by oral

administration of ethanol extract of has been recorded (Rao *et al*., 2012). Badar (1991) reported that aqueous and ethanolic extract of *M. azedarach* prepared from flowers and berries at the dose of 1500 mg/kg is non-toxic by oral route of administration with 395mg/kg and 700 mg/kg LD50 value of flowers and berries, respectively.

In the present study, there were no apparent sign of toxidrome after administration of extracts. No change in behavior was noticed and no loss in weight was also observed. There was no significant difference in hematological parameters of control, vehicle and test group of extract from both plants (Table IV). In the case of biochemical parameters, raise in ALT and AST levels was noted in the test group administered with ethanolic extract of *A. indica* while no change in any biochemical parameter was observed in test group administered ethanol extract of *M. azedarach* in comparison to the control and vehicle group (Table IV). The results revealed that the use of *M. azedarach* did not cause any deleterious effect on the functioning of liver and kidney. However, *A. indica* showed slight toxicity to liver. Hence the results suggested that the use of 5000 mg/kg/day of ethanol extract from *M. azedarach* is more safe than ethanol extract from *A. indica*. However further experiments i.e. chronic toxicity, genotoxicity should be conducted to evaluate the safely of *M. azedarach* and *A. indica* for prolonged use.

Braga *et al*. (2021) have reported that *A. indica* extracts has no or low acute toxicity in mammals depending upon the part of the plant used for extract preparation. Ashafa *et al*. (2012) also reported that ethanolic extract of *A. indica* from stem bark is not safe to use orally at the dose of 50 mg/kg, 100, 200 and 300 mg/kg as it raised the serum levels of globulin, bilirubin and cholesterol, significantly.

Table IV. Effect on hematological and biochemical parameters by oral administration of 5000 mg/kg/day ethanol extract of *A. indica* and *M. azedarach* for 3 weeks.

Parameters	Control group (n=3)	Vehicle group (n=3)	Sample group A (*A. indica*) (n=3)	Sample group B (*M. azedarach*) (n=3)
Biochemical parameters				
Total bilirubin (mg/dl)	0.3±0.03	0.31±0.01	0.3±0.014	0.3±0.017
ALT (U/L)	43.67±1.45	46.34±1.20	79.67*±0.88	46.67±1.20
AST (U/L)	80±1.73	83±2.0	187.66*±2.89	82.67±2.52
AP (U/L)	133±1.0	130±1.15	134±1.67	132±1.46
Albumin (g/dl)	4.3±0.1	4.2±0.11	2.33*±0.09	4.2±0.06
Globulin (g/dl)	1.47±0.14	1.46±0.09	1.5±0.06	1.5±0.1
Blood urea (mg/dl)	39.16±0.71	38.73±0.89	39.02±1.28	38.16±1.12
Serum creatinine (mg/dl)	0.52±0.09	0.54±0.06	0.54±0.03	0.52±0.04
Hematological parameters				
WBCs count (10^9/L)	3.93±0.042	3.84±0.03	4.00±0.06	3.91±0.06
Total RBC (million/mm^3)	9.05±0.13	9.38±0.05	9.02±0.13	9.41±0.06
Hemoglobin (g/dl)	12.66±0.40	13.45±0.28	12.83±0.20	12.66±0.52
HCT(PVC) (%)	48.1±0.74	47.89±0.19	48.1±0.305	47.8±0.36
MCV (Fl)	56.21±0.24	55.79±0.15	56.33±0.26	56.06±0.14
MCH (Pg)	15.4±0.17	15.26±0.20	15.5±0.11	15.33±0.14
MCHC (g/ dl)	27.48±0.27	27.5±0.26	27.33±0.26	27.40±0.26
Platelets (/mm^3)	996000±0.20	991666.67±0.40	1003333±0.73	994000±0.12
Lymphocytes (%)	77.53±0.27	75.86±0.24	76.6±0.31	77.23±0.34
Neutrophilis (%)	23.9±0.21	23.23±0.49	23.63±0.45	23.74±0.39

Data is expressed as mean ± standard error of the mean (SEM) and analyzed by one-way ANOVA, followed by Dunnett's test ($P < 0.05$ indicated by *) as compared to respective parameter value of control group. ALT, alanine amino transaminase; AST, aspartate amino transferase; AP, alakaline phosphate; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

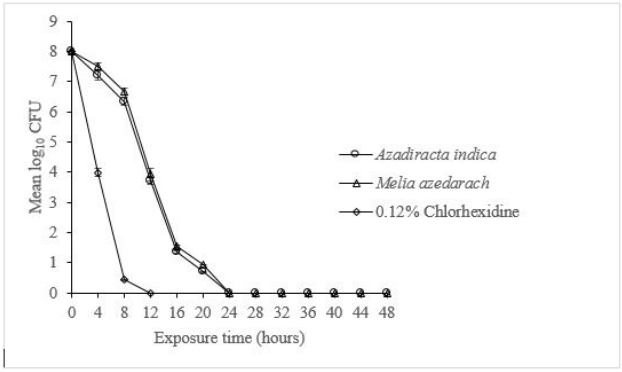

Fig. 2. Time kill kinetic of ethanol extracts of *A. indica* and *M. azedarach* at 1 x MIC. Results are mean of three independent experiments. Error bars are showing the S.E of mean value. Significant reduction in growth was recorded after 12 h of exposure to the ethanol extracts at p-value< 0.0001 after analyzing the data by one-way ANOVA followed by Dunnett's test.

Minibarcodes based marker for identification

As *A. indica* and *M. azedarach* differ in therapeutic efficiency against *P. gingivalis*. Therefore, for quality assurance and standardization of the products against *P. gingivalis* for which *A. indica* or *M. azedarach* has to be used, it is essential to accurately identify both plants. In Indo-Pak continent, *A. indica* and *M. azedarach* both are erroneously used/ sold under same vernacular name neem by many herbalist, local communities and herb sellers due to their morphological similarity (Sultana *et al*., 2011). Even *Melia dubia* is also listed as synonym of *M. azedarach* about while Siviraj *et al*. (2018) reported after DNA barcode analysis that both *Melia dubia* and *M. azedarach* are distinct species. In the present study, for identification of *A. indica* and *M. azedarach*, minibarcodes were developed. Minibarcodes were preferred over the DNA barcode analysis to make the identification more accurate, simple, fast and inexpensive and to avoid the post PCR steps of DNA barcoding such as purification, sequencing and data analysis. With minibarcodes, identification is possible in single step PCR and amplification of minibarcode than full length DNA barcode is more successful in processed plant material (Srirama *et al*., 2014). In the present study, DNA barcode from trnH-PsbA locus was amplified (Fig. 3) from each 20 DNA samples of each plant collected from different cities of Pakistan and sequences were analyzed after sequencing. The sequences showed 100% homology with the available trnH-PsbA sequences of *A. indica* and *M. azedarach* through NCBI BLAST. The trnH-PsbA barcode sequence of *A. indica* and *M. azedarach* were aligned through CLUSTAL omega (Fig. 4). The species specific primers (Table V) were designed from the variable regions to amplify the minibarcodes from each plant of length 275 bp of *M. azedarach* and 315 bpof *A. indica* (Fig. 5A, B). The results showed that markers developed were highly species specific as no cross amplification was observed even no amplification was observed with randomly selected plant species (data not shown).

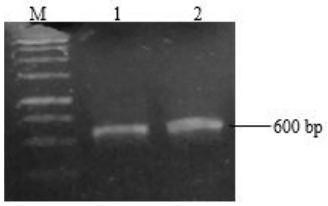

Fig. 3. Amplification of trnH-PsbA barcode of *M. azedarach* (lane 1) and *A. indica* (lane 2); lane M, DNA marker.

```
M. azedarach    CACGAATCATTATTTTTTTATCTTACTTATTTATCAAAATAGAAATGAAGATCGAAGAGG          60
A. indica       --CGAATCATTATTTTTTTATCTTACTTATTTATCAAAATCGAAGGGAATATCGAAAAGA          58
                  ************************************ ***  *** ****** **

M. azedarach    CATAAAATTACAACTTTTCTCTTTTCTTTTACTTTTATATTAAATATATAAAATGTTGTA          120
A. indica       CATAAAATTACAACTTTTCTCTTTTCTTTTACTTTTATATTAAATT---------AAGTC          109
                ************************************************        **

M. azedarach    TAAATTTTTAAAATTGGAATCGAATTAATAAAATTCGAATGTTATTAGTGTTATTAAAAC          180
A. indica       TAAAATTTAAAAATTAGAATCGAATTAATAAAATTCGAATGTTATTAGTGTTATTAAAAC          169
                **** *** ****** ************************************************

M. azedarach    ATAATTAATATTATTATATTGGCTATTGGCACATAACAAATATTAACGTATAACTATAAC          240
A. indica       ATAACTAATATTATTATATTAGCTATTGGCACATAACGAATATTAACGTATAACTCTAAC          229
                **** *************** *************** ****************** ****

M. azedarach    CAAAATATTATGCTAA-ATCAAAGCAAAAGCATAAAAATATGTTTCAGAAAAAAAGGATG          299
A. indica       AAAAATATTTATAGTAAAGCAAAACAAAAGCATAAAAATACGTTTC-------------          275
                 *********     * * ***** ***************** *****

M. azedarach    TAAATAAAAAACTACT      315
A. indica       ----------------      275
```

Fig. 4. Sequence of minibarcodes of *M. azedarach* and *A. indica* aligned by Clustal omega. * sign represent the homology between the sequences and underlined area shows the region of species specific markers developed for *M. azedarach* and *A. indica*.

Table V. Species specific markers of *M. azedarach* and *A. indica.*

Marker name	Sequence	Minibarcode length (bp)
MA-PsbA	F 5'CACGAATCATTATTTTTTTATCTTACTTATTTATCAAAATA 3'	275
	R 5' AGTAGTTTTTTATTTACATCCTTTTTTTCTGAAACA 3'	
AZ-PsbA	F 5' CGAATCATTATTTTTTTATCTTACTTATTTATCAAAATC 3'	
	R 5' GAAACGTATTTTTATGCTTTTGTTTTGCTTTACT 3'	315

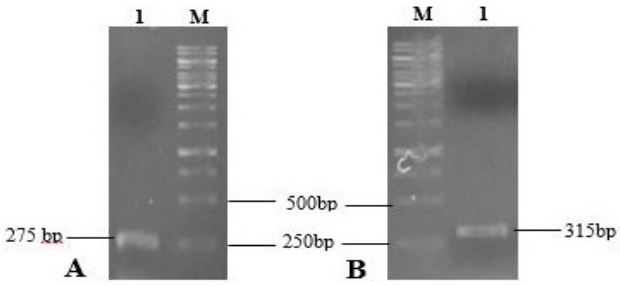

Fig. 5. Amplicons of minibarcodes using species specific markers of *M. azedarach* and *A. indica*. A, minibarcode of *M. azedarach* of length 275 bp (lane 1); B, minibarcode of *A. indica* of length 315 bp (lane 2); lane M, DNA marker.

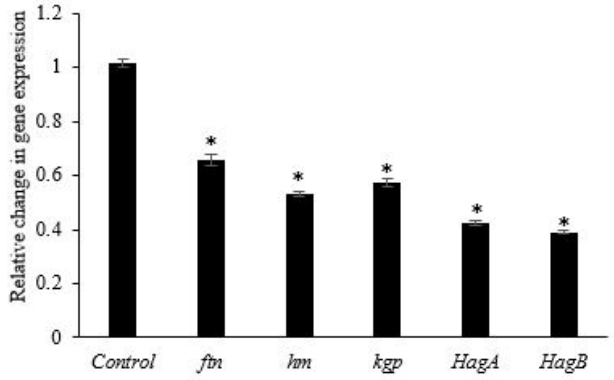

Fig. 6. Gene expression analysis of virulence factor genes of *P. gingivalis* by RT-PCR. Significant down regulation in expression of all the studied genes was recorded at transcription level where p-values are less than 0.001 (indicated by *). The data was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Error bars indicating the standard error where n=3).

Gene expression analysis of virulence genes

The change in the expression of the genes involved in the virulence of *P. gingivalis* after treatment with *M. azedarach* ethanol extract at 1xMIC was studied by real-time PCR. The results showed that expression of all the studied genes was down regulated after treatment with *M. azedarach* ethanolic extract (Fig. 6). The major virulence of *P. gingivalis* in peridontal diseases is due to the gingipains which consist of three cysteine proteases, two are arginine specific (*rgpA, rgpB*) and one is lysine specific (*kgp*). Gingipains affect the host cell by detaching epithelial cells from connective tissue of gingiva, by altering cell signal transduction and by degrading thedifferent proteins of host such as integrin, collagen, cytokines and complement system proteins (Baba *et al*., 2001; Takki *et al*., 2005; Nakayama *et al*., 2015). Heme is required by *P. gingivalis* as iron source and to survive in host cell. The genes of hemagglutination (*HagA, HagB*) and hemolysin (*hm*) play role in acquisition of heme from host cells and adherence of *P. gingivalis* to gingival epithelial cells (Smalley and Olczak, 2017). The ferritin (ftn) helps the *P. gingivalis* to survive in iron deficit environment (Ratnayake *et al*., 2000). The significant reduction in the expression of virulence genes gives some insight about the mechanism of action of ethanol extract of *M. azedarach* against *P. gingivalis*. However, further studies are required to fully understand the mechanism of action of *M. azedarach* against *P. gingivalis*.

CONCLUSION

A. indica, which is traditionally used for periodontal diseases has the chances of adulteration by its allied plant species *M. azedarach*. Therefore, the present study explored the therapeutic potential of *M. azedarach* against *P. gingivalis* and also its *in vivo* safety profile in mice in comparison to *A. indica*. The results showed that *M. azedarach* also has antibacterial activity against *P. gingivalis* with effectivity half to the *A. indica*. However, oral administration of *M. azedarach* ethanolic extract from leaves in mice attributed higher safety profile than *A. indica*. Hence, the present study suggests the *M. azedarach* can also be considered as potent candidate against *P. gingivalis* infection with improved safety profile. Additionally, the development of minibarcodes to identify the either plant by an easy single step PCR, further improve the standardization and quality assurance of product used against *P. gingivalis*.

DECLARATIONS

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IRB approval

The experimental design for experimental animal use was approved by the local Biosafety and Bioethics Committee at the University of the Punjab, Lahore, Pakistan.

Ethical statement

We hereby confirm that the study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Statement of conflict of interest

The authors have declared no conflict of interest.

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