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Antimicrobial Potential of *Juglans regia L* (Walnut) Against Oral Pathogens Associated with Caries and Periodontitis

Azhar Rafique[1*], Muhammad Sharjeel Arshad[2], Nadeem Ali[3] and Md. F. Kulyar[4]

[1]Department of Zoology, Faculty of Life Sciences, Government College University Faisalabad; [2]Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan; [3]Center of Excellence in Environmental Studies, King Abdulaziz University, Jeddah, Saudi Arabia; [4]Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, LT-08406 Vilnius, Lithuania
*Correspondence: azharrafique@gcuf.edu.pk

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ABSTRACT

The non-transmissible diseases dental caries and ulatrophia pose a huge burden on human beings. Exploration for economical, easily accessible, harmless and substitute therapeutics development for the oral and dental health care is required. Natural products are used as traditional medicines from ancient times in Ayurvedic system of medicines. (walnut) is a type of plant that is commonly present around the Globe. The current research explores the antibacterial potential of three different solvent extracts including aqueous, acetone and methanol contrary to *Prevotella intermedia, Streptococcus mitis* and *Streptococcus mutans.* Vero cell line was used to determine the cellular cytotoxicity (CC50). For the phytochemical analysis all three extracts were evaluated through HPLC (High Performance Liquid Chromatography). The findings of current experiment showed that the aqueous extract of have considerably higher antibacterial activity by measuring the diameter of zone of inhibition (20.00±0.58mm, 17.00±0.58mm) trailed by acetone and aqueous extracts counter to *S. mutans*, *S. mitis*, respectively. The methanolic extract proved statistically significant antibacterial activity at 160 mg/ml with inhibition zone (15.67±0.33mm) followed by acetone and aqueous extract against *P. intermedia.* The MIC (Minimum Inhibitory Concentration) (20mg/ml of aqueous extract against *S. mutans*; 5mg/ml of aqueous extract *S. mitis* and 10 mg/ml of methanol against *P. intermedia*). Highest quantity of phenolic acids was showed by methanol extract through HPLC while aqueous extract has considerably higher CC_{50} and for therapeutics it's the safest among methanolic and acetone extract.

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INTRODUCTION

Oral health is integral and vital for the human health, prosperity and well-being of life. Furthermore, the general health of an individual is interlinked with oral health; non-transmissible chronic diseases and oral cavity infections share "communal risk aspects" (Conway et al. 2013). Oral illnesses are most common factor that affecting the oral health, mostly inflammation of teeth (periodontitis), dental decay and rarely by orofacial cracks and pain, oral cancer and HIV lesions (Jin et al. 2016). Cavities and tooth decay are complex multifactorial infections with tooth plaque as their key reason (Shafiei et al. 2012). Dental caries is ubiquitous process caused by a confined chemical dissipation of the tooth surface prompted by acid produced due to the dental plaque exposed frequently to sugars (Fejerskov et al. 2015). Ulatrophia includes gingivitis and periodontitis are inflammatory infections of dental biofilm. Vital risk factor related to the inappropriate and damaging inflammatory response is the accretion of dental biofilm under the gingival margin (Chapple et al. 2015; Jepsen et al. 2017). Dental caries and periodontitis are caused by the imbalance of the dental biofilm (Teanpaisan et al. 2017). Dental plaque or biofilms are formed by the adherence and pellicle formation (thick layer of glycoproteins) on to the tooth surface that is instantly formed on clean surface. Ultimately, the sticky and soft deposition that is apparently observed on tooth surfaces clinically termed as dental plaque (Manjia et al. 2018). Removal of bacterial plaque biofilms remains a challenge for health care centers. Oral microflora approximately comprises of 700 microbial species and 19,000 genotypes (phylotypes) (Keijser et al. 2008). In natural microflora of human body, the most diverse range of microbes present in oral cavity that deal with more than 1000 species (Manji et al. 2018). Endogenous bacteria produce organic acid that decreases plaque pH, which leads to demineralization of the tooth surface (Kim et al. 2017). Various factors including suitable pH, temperature and moist environment enhances the bacterial growth (Marsh et al. 2011).

Periodontal diseases and tooth decay (caries) are not caused by specific pathogens. The mechanism of interaction between microbiota-environment and host is much more complex (Colombo and Tanner 2019). Worldwide prevalence of dental caries is high and untreated dental caries that effecting human beings frequently (GBD 2016). As per recent global estimations, untreated caries in dentine in primary teeth and in permanent teeth are 621 million in children and 2.4 billion in all age group population respectively (Kassebaum et al. 2015). Globally almost 743 million people are affected by severe periodontitis (Jepsen et al. 2017). Various types of bacteria can grow and persist in the periodontal diseases and gingival crevice (Newman et al. 2006). Supragingival and subgingival are the two types of plaque. Supragingival portion possess mostly gram-positive bacteria including *Streptococcus sanguinus, Streptococcus mutans*, *Streptococcus salivarius, Streptococcusmitis* and *lactobacilli*. Gram negative bacteria (*Actinomycetem comitans*, *Actinobacillus, Fusobacterium nucleatum Porphyromon asgingivalis*, spirochetes such as *Treponema denticola* and *Prevotella intermedia* primarily resides in subgingival plaque (Aas et al. 2005; Paster et al. 2001).

Medicinal plants produce different kind of metabolites and antimicrobial components that can be effective for the treatment of various infections and also have long term beneficial health effects. Secondary metabolites effectively have therapeutic actions in humans. From beginning to till current era almost one third of the total plant species are used for the treatment of different diseases due to the presence of secondary metabolites (Agbor and Naidoo 2015). For the treatment of different oral diseases and bacterial resistance against antibiotics, an effective therapeutic agent (secondary metabolites) of plants is being used in developing countries all over the world. Plants based treatment is extremely effective, economical, easily available and safe for the immune-compromised patients as an alternative to other preventive measures (Palombo 2011). Herbal products have antiviral, antifungal, antibacterial and anti-inflammatory activities and have found its way into dental medicine (Karadaglıoglu et al. 2019). Herbal products can inhibit the biofilm formation and reduce the microbial adhesion which is the main cause of dental plaque and caries (Barnes and Arnold 2018).

Walnut tree (*Juglan regia L.*) bark is a main source of Derum is one of the natural "chewing sticks" and also mainly used in cosmetics products as a dye and as a tooth brush (Kadum and Al-Waheb 2010). The family Juglandaceae, genus Juglans (family) includes numerous species and it is present worldwide. Walnut is its prominent member, constituting a substantial species of plants which are commonly present in the moderate climate areas and cultivated at commercial scale in the Asia, western South America, United States and southern and central Europe (Pereira et al. 2007).

The particular characteristics of walnut plant made it as effective substitute to antibiotics, chemical preservatives and antioxidant activity. Recent research evaluated the antimicrobial activity of phenolic extracts of walnut as well (Sousa et al. 2006; Muzaffer and Paul 2018). Various extract of medicinal plants has been reported as inhibitor of dental biofilm by reducing the adherence of pathogens to the tooth surface, that's leads to the progression of dental decay (Jain et al. 2015).

Walnuts comprises of different ploysaturated fatty acids, amino acids, phenolic compounds, dietary fatty acids and minerals (Kavuncuoglua et al. 2018). Folic acid, β-sitosterol, Gallic acid, ascorbic acid, regiolone and quercetin-3-α-L-arabinoside are present in stem bark. Several studies show the antioxidant, antifungal and antibacterial activity of this plant (Noumi et al. 2010, Zakavi et al. 2013).

The current experiment estimates the determination of phenolic acids of plant extract through High performance liquid chromatography (HPLC) and antibacterial effect against oral microorganisms associated with periodontitis and dental caries. Cytotoxicity of different solvent extracts was also examined to find the CC_{50}.

MATERIAL AND METHODS

Acquisition of Medicinal plants

Was attained from local source (Faisalabad city). Plants were verified from Botany Department University of Agriculture, Faisalabad, Pakistan.

Preparation of plant extract

Bark was dried under shade and grinded by a grinding mill. Total 20 gram of sample was extracted in deionized water 200ml, acetone 70% and methanol 70%, respectively (Sultana et al. 2007). Extract was filtered by using vacuum filtration assembly using Whatman No. 1 filter paper. Through rotary evaporator filtrate was dried and stored at 4°C for further use.

Bacterial Strains

Three bacterial strains including *S. mitis* (ATCC 6249), *S. mutans* (ATCC 25175) and *P. intermedia* (ATCC 25611) were procured from Institute of Microbiology, University of Agriculture, Faisalabad. Salivarius agar (Himedia, India) was used as selective medium for *S. mutans* and *S. mitis* at 37^{o}C for 24 hrs under aerobic conditions. Basal agar (Himedia, India) was used for the anaerobic growth of *P. intermedia,* and incubated under anaerobic conditions for 72 hrs.

Bacterial inoculum Preparation

McFarland (0.5) standard was used for the adjustment of bacterial suspension through spectrophotometer.

Evaluation of extract as an antimicrobial agent

For the assessment of antimicrobial activity of different extracts of agar well diffusion method was used with minor modifications (Andualem et al. 2014). For the aerobic and facultative anaerobic bacterial strains, Mueller Hinton agar and basal agar were used, respectively. Broth culture of bacterial strains standardized with McFarland standard and streaked with sterilized cotton swab on agar plates, following this after 30 min by using cork borer wells of 6mm diameter were made on each plate equidistantly. Each well poured with 50µL of the extract solution. Aerobic and facultative anaerobic bacterial plates were incubated aerobically and anaerobically at 37 ºC for 24 hrs and 48 hrs, respectively. After the incubation, zone of inhibition was observed of each type of extract against all the bacterial strains that shows the antimicrobial potential of respective extract. Diameters of the zones were measured in millimeters and all the extracts were assessed in triplicate for the mean value.

Determination of minimum inhibitory concentration (MIC)

For the determination of the minimum inhibitory concentration of plant extract, microdilution method was adopted with minor modifications (Amanullah et al. 2018). Dimethyl sulfoxide (DMSO) 100ul was used as a solvent for dry plant extract. Mueller Hinton broth and anaerobic basal broth were serially diluted (twofold dilution) with plant extract in microtitration plate. Then in 96 well microtitration plate 100 ul standardized inoculum of bacterial strains were added and incubated at 37ºC for 24 hrs. 40 µl of 0.2 mg/ml of P-iodonitrotetrazolium chloride (INT) was added as a bacterial growth indicator in all wells and incubated for 30 min at 37 °C. Hence the change in the color due to the INT was used to evaluate the MIC (Eloff 1998). The well with no color change in medium was confirmed as MIC of sample concentration.

Minimum bactericidal concentration (MBC)

It is the minimum concentration of extract at which the tested bacteria were killed. MBC of various extract determined by taking 10 ul from each well that have no visible growth and poured on the respective agar plates and results were evaluated after 24 hrs incubation at 37°C.

Cytotoxicity assay (MTT)

Microculture MTT method was followed for determination of cytotoxicity of plant extracts. Each well is harvested with 2.0-2.8x10^5 cells and inoculated in 24 well plates. Phosphate buffer saline was used for the washing of cells and after that inoculated with and without the extract. Each sample was inoculated two times. Then, the cells were treated within MTT (Artun et al, 2016) and incubated at 37°C for 48 hours, 20µl of 5 g/l MTT was added to all the well and incubated for 4 hrs. Formazan crystals were solubilized by addition of warm 200 ul DMSO after removal of MTT from each well. The results were examined at 490 nm wavelength. . Control wells cultivated without drugs in same conditions.

Data were calculated as percentage of inhibition by the following formula.

$$\text{Inhibition }\% = \left[100 - \left(\frac{\text{ODt}}{\text{ODs}}\right) \times 100\right]\%$$

HPLC analysis of plants extracts

For the phytochemical analysis of plant extract HPLC method with minor changes was used (San and Yildirim 2010). Initially, mobile phase was goes through degasser DGU-12A, Schimadzu, Japan, for the removal of any kind of bubble. Qualitative and quantitative analysis of sample were done by comparison with standard curves. Different phenolic acids present in plant extract were estimated through suitable mathematical calculations.

Statistical analysis

Statistical Package for Social Sciences Version 17 (SPSS Inc. Chicago, U.S.A) was used for the data analysis.

RESULTS

Antibacterial Effects of Extracts

The various extracts of ***J. regia*** exhibited variable degree of antibacterial activity against oral pathogenic bacteria. All the plant extract tested with different concentrations against all bacterial pathogens. Methanolic extract showed highest significant results against all bacterial strains. Acetone and aqueous extracts observed as less effective as compared to methanolic extract. Tables (1, 2, 3) and Figures (1, 2, 3) explained the results respectively of *S. mitis*, *S. mutans* and *P. intermedia*are.

Table 1: Antimicrobial activity *J. regia* aqueous, acetone and methanol extract against *S. mitis*

Concentration mg/ml	Aqueous (Zone of inhibition in mm)	Acetone (Zone of inhibition in mm)	Methanol (Zone of inhibition in mm)
10	10.00±0.58D	8.33±0.88C	8.33±0.33D
20	11.33±0.94D	8.67±0.67C	9.00±0.58D
40	13.00±1.41CD	9.67±0.88BC	10±1.15CD
80	16.00±1.63BC	10.33±0.67BC	12.67±0.33BC
160	20.00±0.58A	13.00±1.15B	15.00±1.41AB
Vancomycin	16.67±0.33AB	16.67±0.33A	16.67±0.33A
PBS	0±0E	0±0D	0±0E
Overall Mean	12.43±1.36	9.52±1.08	10.24±1.15

Values are mean diameter of inhibition zone in millimeter (mm) ± S.E of three replicate.

The Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). PBS; phosphate buffer saline, vancomycine (30 µg) was used as reference antibacterial compound.

Table: 2: Antimicrobial activity *J. regia* aqueous, acetone and methanol extract against *S. mutans*

Concentration mg/ml	Aqueous (Zone of inhibition in mm)	Acetone (Zone of inhibition in mm)	Methanol (Zone of inhibition in mm)
10	0±0D	0±0D	0±0D
20	8±0.58C	0±0D	8.33±0.88BC
40	9.67±0.88C	8.5±1.41C	10.67±0.88BC
80	12.67±0.67B	10.00±0.58B	11.67±1.2
160	17±0.58A	12±0.58B	13±1.41AB
Vancomycin	16.67±0.33A	16.67±0.33A	16.67±0.33A
PBS	0±0D	0±0D	0±0D
Overall Mean	9.14±1.47	6.67±1.41	8.62±1.35

Values are mean diameter of inhibition zone in millimeter (mm) ± S.E of three replicate.

Table 3: Antimicrobial activity *J. regia* aqueous, acetone and methanol extract against *P. intermedia*

Concentration mg/ml	Aqueous (Zone of inhibition in mm)	Methanol (Zone of inhibition in mm)	Acetone (Zone of inhibition in mm)
10	0±0C	8.33±0.33C	0±0D
20	0±0C	8.33±0.33C	0±0D
40	0±0C	10.33±1.2C	0±0D
80	0±0C	13.67±0.33AB	8.33±0.33B
160	8±0.58B	15.67±0.33A	10.33±0.88AB
Clindamycin	11±0.58A	11±0.58BC	11±0.58A
PBS	0±0C	0±0D	0±0D
Overall Mean	2.71±0.98	9.62±1.05	4.24±1.12

Values are mean diameter of inhibition zone in millimeter (mm) ± S.E of three replicate.

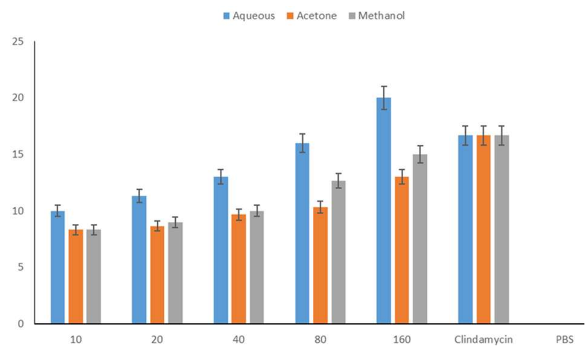

Fig. 1: Graphic presentation of antimicrobial activity of *J. regia* aqueous, acetone and methanol extract against *S. mitis.*

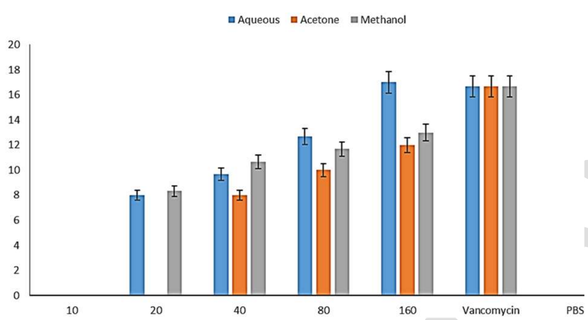

Fig. 2: Graphic presentation of antimicrobial activity of *J. regia* aqueous, acetone and methanol extract against *S. mutans.*

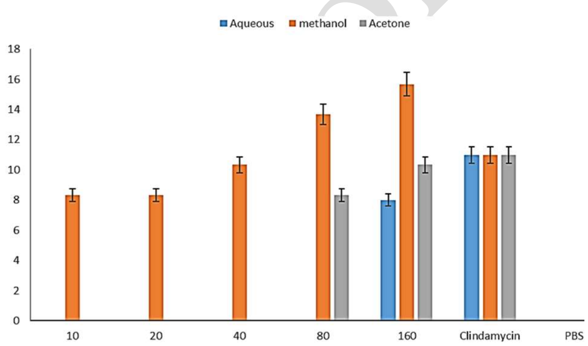

Fig. 3: Graphic presentation of antimicrobial activity of *J. regia* aqueous, acetone and methanol extract against *P. intermedia*.

The Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). PBS; phosphate buffer saline, vancomycine (30 µg) was used as reference antibacterial compound.

The means sharing similar letter in a row or in a column are statistically non-significant (P>0.05) PBS; phosphate buffer saline, Clindamycin (30 µg) was used as reference antibacterial compound.

Minimum Bactericidal Concentration (MBC) and Minimum inhibitory concentration (MIC): MIC and MBC were examined by using different concentrations of all three extract of (Table 4 and Figure 4).

Table 4: Minimum inhibitory concentration (MIC mg/ml) of different solvent extracts of *Juglans regia* against *P. intermedia*, *S. mitis* and *S. mutans*

Microorganism	Aqueous	Acetone	Methanol
Strepococus mutans	20.00±0.00b	40.00±0.00a	20.00±0.00b
Strepococus mitis	5±0.00a	10.00±0.00b	10.00±0.00b
Prevotella intermedia	160.00±0.00c	80.00±0.00a	10.00±0.00b

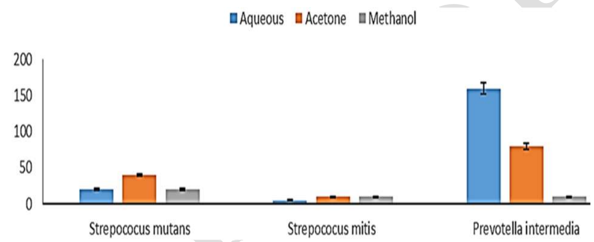

Fig. 4: Graphic presentation of MIC of different extracts of *J. Regia* against bacteria.

The aqueous and methanol extracts showed significantly higher activity (20.00±0.00, 20.00±0.00mg/ml each) followed by acetone extract against *S. mutans*. The aqueous extract showed significantly higher activity against *S. mitis*. The methanol extract was significantly effective than aqueous and acetone extract against *P. intermedia*. Table 5 and Fig. 5 showed MBC values of tested bacteria. The MBC of all extracts was higher than MIC against *S. mitis*. The MBC of methanolic, aqueous and acetone were 20.00±0.00 mg/ml 320.00±0.00, 160.00±0.00 correspondingly than MIC against *P. intermedia*.

Table 5: Minimum bactericidal concentration (MBC mg/ml) of different solvent extracts of *Juglans regia* against *P. intermedia, S. mitis* and *S. Mutans*

Microorganism	Aqueous	Acetone	Methanol
Strepococus mutans	20.00±0.00b	40.00±0.00a	40.00±0.00a
Strepococus mitis	10.00±0.00c	20.00±0.00a	20.00±0.00a
Prevotella intermedia	320.00±0.00c	160.00±0.00a	20.00±0.00b

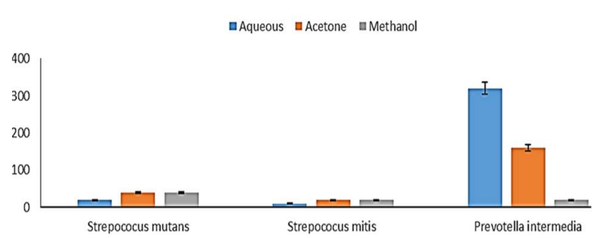

Fig. 5: Graphic presentation of MBC of different extracts of *J. Regia* against bacteria.

Cytotoxicity assay

Dose dependent cytotoxicity of all three solvent extracts of were observed to Vero cells. Cell growth was suppressed with the higher concentrations of extracts inhibit the more cells. Table (6, 7, 8) showed the inhibitory percentage of methanolic, acetone and aqueous extract respectively.

HPLC analysis of *A. nilotica*

For the determination of phenolic acids in all three extracts of, HPLC was performed and methanolic extract has higher concentration of phenolic acids than aqueous and acetone extracts. Concentration of phenolic acids of all three extracts given in Table 10, Fig. 7.

Cellular Cytotoxicity (CC50)

Regression analysis was used to calculate the cellular cytotoxicity (CC50) from the observed data of inhibition dose response. The methanolic, acetone and aqueous extract showed CC_{50} results as 278.43±29.77 µg/ml, 632.78±24.38 µg/ml and 929.65±47.48 µg/ml, respectively. Aqueous extract shows significantly higher CC_{50} than acetone and methanolic extract. Table 9 and Fig. 6 explain the CC_{50} results.

Table 6: Percentage inhibition of Vero cells by *J. regia* (aqueous)

Concentration µg/ml	Absorbance	%age inhibition	t vs control
Control	0.97±0.06	-	-
156.25	0.66±0.03	32.45±3.03	10.491
312.5	0.49±0.04	49.45±4.53	10.807
625	0.4±0.04	58.45±4.17	13.928
1250	0.35±0.03	64.39±3.5	18.314
2500	0.24±0.03	74.83±2.99	24.927

Assay were performed in triplicate Mean±SE.

Table 7: Percentage inhibition of Vero cells by *J. regia* (methanol)

Concentration µg/ml	Absorbance	%age inhibition	t vs control
Control	0.97±0.06	-	-
156.25	0.63±0.02	35.47±1.67	20.813
312.5	0.51±0.01	47.6±0.85	55.652
625	0.38±0.02	61.2±2.35	25.876
1250	0.34±0.02	64.9±2.33	27.755
2500	0.28±0.02	71.53±2.44	29.223

Assay were performed in triplicate Mean±SE.

Table 8: Percentage inhibition of Vero cells by *J. regia* (acetone)

Concentration µg/ml	Absorbance	%age inhibition	t vs control
Control	0.97±0.06	-	-
156.25	0.63±0.01	34.99±1.19	28.776
312.5	0.49±0.01	49.66±0.62	78.693
625	0.37±0.01	61.4±1.49	41.027
1250	0.36±0.01	62.98±0.54	116.734
2500	0.28±0.02	71.5±2.15	33.102

Assay were performed in triplicate Mean±SE.

Table 9: CC_{50} µg/ml of different extracts of *J. regia*

Aqueous	568.35 ± 25.33a
Acetone	481.05±79.68c
Methanol	492.98 ± 87.38b

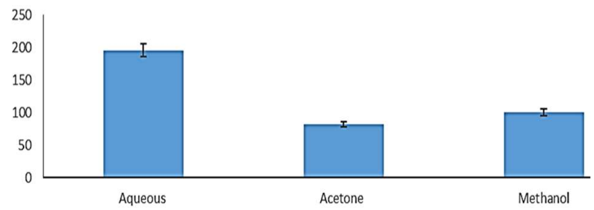

Fig. 6: Graphic presentation of CC_{50} of different *J. regia* extracts.

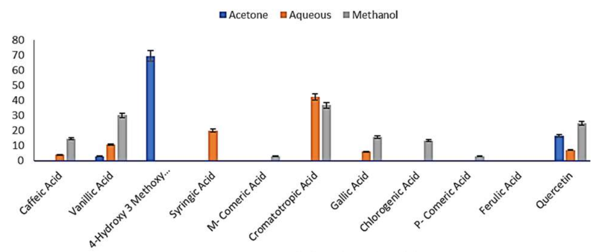

Fig. 7: Graphic presentation of phenolic acid in different extracts of J. regia.

DISCUSSION

Globally among many of major health problems oral diseases are still a major concern (Dash et al. 2014). Other than dental cries and periodontal diseases some other including oral cavity lesions, cancer of oral cavity and throat tissues also have great importance (Petersen 2003). Oral hygiene has great impact in general wellbeing of humans. Quality of life and whole-body systems other than craniofacial complex are deeply concerned with oral health. Treatment for the oral diseases and caries is much expensive in many countries (Sheiham 2005). For the treatment of oral diseases many natural and herbal remedies are being practiced since long time. Herbal and medical plants are very effective against many diseases. Researchers and health care authorities still working on many natural products against oral pathogens (Palombo 2011). Secondary metabolites of plants including phenolic compounds, are globally present in different vegetables, fruits and dietry products. Mainly phenolics are divided into three main groups including phenolic acids, flavonoids and tannins (Borges et al. 2013). The current study was conducted to evaluate the antibacterial activity of different extracts (methanolic, aqueous acetone) extract of *against*

Table 10: Phenolic of different solvents of *J. regia* (ppm)

Solvent	Caffeic Acid (ppm)	Vanillic Acid (ppm)	4-Hydroxy 3 Methoxy benzoic Acid (ppm)	Syringic Acid (ppm)	M-Comeric Acid (ppm)	Cromatotropic Acid (ppm)	Gallic Acid (ppm)	Chlorogenic Acid (ppm)	P- Comeric Acid (ppm)	Ferulic Acid (ppm)	Quercetin
Acetone	0.00±0.00C	20.80±0.15 B	69.50±0.36 A	0.00±0.00B	0.00±0.00 B	0.00±0.00C	0.00±0.00C	0.00±0.00B	0.00±0.00 B	0.00±0.0 0	16.51±0.17 B
Aqueous	3.80±0.15B	10.70±0.36 C	0.00±0.00B	19.90±0.06 A	0.00±0.00 B	42.30±0.35A	5.90±0.10B	0.00±0.00B	0.00±0.00 B	0.00±0.0 0	7.03±0.08C
methanol	14.50±0.32 A	30.02±0.65 A	0.00±0.00B	0.00±0.00B	2.90±0.21 A	36.70±0.53B	15.60±0.26 A	13.26±0.18 A	2.80±0.25 A	0.00±0.0 0	24.84±0.23 A

oral pathogenic bacteria linked with periodontal diseases and caries.

The leaves are an easy to get remedy for prevention of oral diseases (Sharafati-Chaleshtori et al. 2011). Phenolic acids and flavonoids are extensively used in various pharmaceutical products due to their antioxidant activity. (Zheng and Wang 2001). Moreover, antibacterial potential of phenolic compounds has been already confirmed by different researchers worldwide because of this property phenolic extract of medicinal plants are considered as alternative to the antibiotics (Oliveira et al. 2008). Walnut is considered as an excellent source of antimicrobial compounds. Against *S. mutans* aqueous extract of evaluated statistically significant antimicrobial activity (17.00±0.58mm) (MIC 20 mg/ml) followed by methanol and acetone extracts. While against *S. mitis,* aqueous extract (20.00±0.58mm) was significantly effective too that followed by methanol and acetone, but against *P. intermedia*, methanol extract showed significant results (15.67±0.33mm). that followed by acetone and aqueous extract. The findings of current study were in accordance with the results of (Darmani et al. 2006; Pereira et al. 2007) which reported that aqueous extract of showed significant growth inhibition of oral pathogenic bacteria *Lactobacillus casei, S. mutans*, *S. salivarius* and *Actinomyces Viscosus*. The antibacterial including gram positive and gram negative, and antifungal potential of were evaluated by Alkhawajah (1997). Gram-negative bacteria have outer membrane that is made up of lipopolysaccharides covering, it can prevent hydrophobic compounds to diffuse into the cell but phytochemicals can pass easily through gram positive bacterial cell wall and also disturb active transport that results in coagulation of cells (Burt 2004). The MIC range from 20-40 mg/ml against *S. mutans*. MIC of Aqueous and methanol extract of *was* low (20 mg/ml) as compared to acetone extract that 40 mg/ml. The lower MIC was an indication of effectiveness of the extract. The MIC against *S. mitis* was 5.00 mg/ml of aqueous followed by acetone and methanol which was 10 mg/ml of each extract. The MIC against *P. intermedia* was 10 mg/ml of methanol followed by acetone 80 mg/ml than aqueous 160 mg/ml. the MBC value of three solvents were similar to MIC against *S. mutans*. MBC value of extracts were higher than the MIC value against *S. mitis* and *P. intermedia*. The aqueous and acetone extract of were tested against salivary flora as an antibacterial agent by (Deshpande et al. 2011; Jagtap and Karkera 2000). Various extract were also examined against Vero cells to estimate CC_{50}. Aqueous extract showed significantly higher (568.35±25.33 µg/ml) CC50 than the methanolic (490.98±25.33 µg/ml) and acetone (481.05±79.68 µg/ml) extract. The percentage of inhibition of cells were determined by dose response curve. Various extracts of showed no statistically significant difference between percentage inhibitions of cells. Ethanolic extract of responded differently with different cell lines. The CC_{50} of ethanol extract was 100 $\mu g mL^{-1}$. The different solvents showed divergent results that was in accordance with the finding of Chaieb et al (2013). The HPLC analysis of various extracts of were quite variable. Methanolic extract of has highest phenolic acids followed by aqueous and acetone extract. The findings of our HPLC results conform with the previous finding which reported that extraction efficiency of phenolic compounds depends on solvent used. Phenolic compounds were found to be high in number in methanolic extract as compared to other solvents (Cosmulescu et al. 2010) and Pereira et al. (2007) reported that isolation and identification of ten phenolic compounds from walnut leaves. The phenols and flavonoids of walnut leaves are likely responsible for the antibacterial activities of the extract derived from (Stampar et al. 2006). Cueva et al. (2010) reported that phenolic acids has good inhibitory effect on intestinal bacteria and microbes in comparison with (-) epicatechin and (+) -catechin.

Conclusion

Methanol proved to be the best solvent for extraction of phenolic acids and its extract is most effective against pathogens under study. On the basis of CC_{50}, aqueous extract is the safest among the three solvent extracts to be used for therapeutic purposes. The results of present study suggest that ***J. regia*** may be used alone or in combination with other plant extracts in mouth wash or tooth paste to prevent oral diseases.

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Ethics Statement: All the research procedures were conducted according to guidelines regarding use of animal for research purpose.

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