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Effect of extraction solvent system on the antimicrobial, antioxidant and total polyphenol content of the bark of *Pistacia chinensis*

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ABSTRACT – In the current study, the bark of *Pistacia chinensis* was screened for their antibacterial, antioxidant activities, and total polyphenol content in different extraction solvent systems. Extracts from the bark were produced in different extraction solvents like methanol, ethyl acetate, and n-hexane, and tested against different bacterial species using the disc diffusion method. Ethyl acetate extract was more effective against *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Citrobacter freundii*, *Agrobacterium tumefaciens,* and while the methanol extract inhibited the growth of *B.atrophaeus* and *X.oryzae*. Among the tested samples, the maximum zone of inhibition was formed by ethyl acetate extract (69.04% ZI) against *S.typhi*. The n-hexane extracts bark were comparatively less effective against the tested microorganisms. Furthermore, the radical scavenging assay of the extracts revealed that methanol extract exhibited strong antioxidant potential (79%), followed by ethyl acetate and n-hexane extract at 250 µg mL^{-1}. The bark of the plant is also a good source of naturally occurring polyphenol. The polyphenol assay of the different extracts revealed the presence of a high concentration of total polyphenol in methanol (517.25 mg g Gallic acid $equivalent^{-1}$) extract followed by ethyl acetate (393.97 mg g GAE^{-1}) and n-hexane extract (378.7 mg g GAE^{-1}). However, the results of the study did not establish the bridge between total polyphenol and antioxidant activity of the extracts.

Keywords: antimicrobial agents, antioxidant, extraction solvent system, polyphenol

RESUMO – Efeito do sistema de solventes de extração sobre o conteúdo antimicrobiano, antioxidante e de polifenóis totais da casca de *Pistacia chinensis*. No presente estudo, a casca de *Pistacia chinensis* foi avaliada quanto às suas atividades antibacteriana, antioxidante e teor de polifenóis totais em diferentes sistemas de extração de solventes. Extratos da casca foram produzidos em diferentes solventes de extração, como metanol, acetato de etila e n-hexano, e testados contra diferentes espécies bacterianas usando o método de difusão em disco. O extrato de acetato de etila foi mais efetivo contra *S. typhi*, *P. aeruginosa*, *E. coli*, *C. freundii*, *A. tumefaciens*, enquanto o extrato metanólico inibiu o crescimento de *B. atrophaeus* e *X. oryzae*. Entre as amostras testadas, a zona máxima de inibição foi formada pelo extrato de acetato de etila (69,04% ZI) contra *S. typhi*. Os extratos n-hexânicos da casca foram comparativamente menos eficazes contra os microrganismos testados. Além disso, o ensaio de sequestro de radicais dos extratos revelou que o extrato metanólico exibiu forte potencial antioxidante (79%), seguido pelo acetato de etila e extrato n-hexano a 250 µg mL-1. A casca da planta também é uma boa fonte de polifenol natural. O ensaio de polifenol dos diferentes extratos revelou a presença de uma alta concentração de polifenol total no extrato metanol (517,25 mg g equivalente-1 de ácido gálico) seguido de acetato de etila (393,97 mg g GAE-1) e extrato n-hexano (378,7 mg gGAE-1). No entanto, os resultados do estudo não estabeleceram a ponte entre o polifenol total e a atividade antioxidante dos extratos.

Palavras-chave: agentes antimicrobianos, antioxidante, sistema de solvente de extração, polifenol

INTRODUCTION

Infectious diseases are the world's major threat to human health and a serious concern for health professionals and common people around the world (WHO 2019). Microbial infections are one of the leading causes of human death worldwide especially in developing countries (Chokshi *et al.* 2019). Among these infections, bacteria are the main source of causing simple and complex infections in humans. Antibiotics are the most commonly used as antimicrobial agents in developing countries. Due to the continuous and nonspecific use of antibiotics, the microorganisms are developing resistance to these agents (Ventola 2015, Alder *et al.* 2010). The development of MDR strains of microorganisms is becoming a challenge to the researcher. In this context, the bioactive compounds of natural origin have got incredible accomplishments in serving as a guidepost for the discovery and development of novel antimicrobial agents. Moreover, the antibiotic of plant origin is safe and friendly to nature (Bernardini *et al.* 2018, Atanasov *et al.* 2015).

Cellular components are very sensitive to free radicals (Engwa 2018). These reactive oxygen species badly affected the nucleic acid-protein lipids and thus leading to different complex physiological abnormalities including cardiovascular disease inflammation and aging etc (Katerji

et al. 2019, Patel *et al.* 2014, Basma *et al.* 2011, Hamid *et al.* 2010). The use of vegetables and fruits in the diet is linked with a lower risk of cancer and stroke reported in the previous studies (Wallace *et al.* 2020, Kim *et al.* 2019). This protective effect against the complex physiological abnormalities is due to the scavenging activity of the antioxidants found in the different parts of medicinal plants.

Pisticia chinensis locally called *shnai* in Pakistan is very important for its application in folk medicine belongs to the genus *Pisticia*. Different biological activities like hypoglycemic, anti-atherogenic, anti-inflammatory and anti-insect have been reported from the different species of *Pisticia* (Mehenni *et al.* 2016, Koutsoudaki *et al.* 2005, Özçelik *et al.* 2005, Mehmet *et al.* 2004). In the current study, the different solvent extracted samples of the bark were evaluated for antibacterial, antioxidant activity, and total polyphenol content.

MATERIAL AND METHODS

Plant material (Bark) of *P. chinensis* was collected from tehsil Bar Chamarkand district Bajaur, Khyber Paktun Khwa, Pakistan, and taxonomically identified by Prof Dr. Ghulam Dastagir, Department of Botany, and University of Peshawar, Pakistan. The specimen was deposited in the herbarium of the Islamia College University Peshawar with Voucher No: 3545IUP.

Crude extracts preparation

The bark of the plant was dried in shadow at room temperature. Dehydrated plant material was crushed to a fine powder through an electric grinder. One hundred and fifty gram (150 gm) of plant powder was put in one liter of the different extraction solvent (methanol, ethyl acetate, and n-hexane). The solution was placed in a shaking incubator for three days. After completion of the shaking period, the slurry was then filtered through Whatman filter paper No 1, and the filtrate was then dried in a rotary evaporator at 40 ºC. After complete drying, the extract was weighed and then stored in sterilized glass vials for testing the antibacterial properties by disc diffusion dilution assay (Khan *et al.* 2017).

Antimicrobial bioassay

Nutrient agar and nutrient broth media were weighted and prepared according to the standard manufacturer protocol. After autoclaving, the media was poured into Petri plates. For inoculum preparation, all bacteria strains (Listed in Tab. 1) were grown overnight in broth for proper standardization. Further, using a sterile spreader, 50 µl standard inoculum was spread on the agar plates. Sterile Whatman filter paper discs (6mm in diameter) were placed on the surface of media plates and then the tested extracts were applied to the discs and incubated the plates in the incubator at 37 °C for 24 h (Khan *et al.* 2018). After the incubation period zone of inhibition was measured and then the antibacterial potential of each extract was calculated in the term of percent zone of inhibition by the following formula after replicating each experiment three times:

$$\% \text{ zone of Inhibition} = \frac{\text{Inhibition zone of sample}}{\text{Inhibition zone of positive control}} \times 100$$

Inhibition zone of positive control

Free Radical Scavenging Activity of the Extracts

The antioxidant activity of the samples was determined by the DPPH assay, previously performed by Mensor *et al.* (2001). Briefly, for DPPH radical scavenging activity the stock solution of each plant extract was diluted 250, 125, 50, 25, 10, and 5 µg mL^{-1}) in methanol. Each extract (2.5 mL) was mixed with DPPH (0.3 mM of 1mL) and incubated at 30 °C for 30 minutes in a shaking incubator. Spectronic 20 was used to measure the absorbance of the solutions at 518 nm. Finally, the percent antioxidant activity (AA %) using the following formula:

$$AA\% = (\text{Abs sample} - \text{Abs control}) / \text{Abs control} \times 100$$

Negative control =1 ml of 0.3 mM DPPH plus methanol (2.5 mL)
Positive control = Solution of gallic acid served as a positive control

Table 1. List of the microorganisms used in the experiment

Name of the species	Gram strain type	Provided by
Pseudomonas aeruginosa	Negative	CBM University of Swat.
Escherichia coli	Negative	CBM University of Swat.
Xanthomonas oryzae	Negative	CBM University of Swat.
Bacillus atropus	Positive	CBM University of Swat.
Citrobactorfreundii i	Negative	CBM University of Swat.
Agrobacterium tumefaciens	Negative	CBM University of Swat.
Salmonella typhi	Negative	CBM University of Swat.
Rhizobacter daucus	Negative	CBM University of Swat.

Polyphenol Quantification

The total polyphenol present in the extract was determined by an optimized protocol of Slinkard and Singleton (1977). The extract (0.5 ml) of 1000µg/ml concentration was mixed with 46 ml of H2O, followed by the addition of one ml of FR reagent (1N) and carefully blended into a volumetric flask. Then 3 mL of sodium carbonate deca-hydrate (2%) was added after three minutes and then incubated for two hours in the shaking incubator. The absorbance of the solution was measured at 760 nm. Similarly, the absorption of various gallic acid concentrations (25, 50, 100, and 200 µg mL^{-1}) was also noted and plotted against the concentration to produce the standard equation of gallic acid. This standard curve equation was used for the estimation of total polyphenol in various samples (Fig. 1).

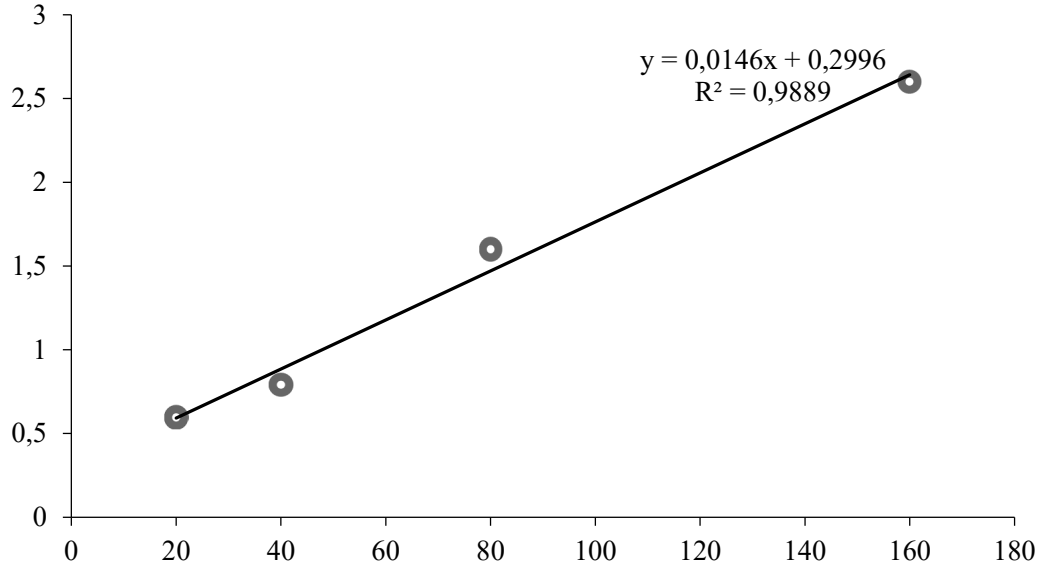

Figure 1. Standard curve for gallic acid y = absorbance, x = concentration of Gallic Acid, R^2 = correlation coefficient.

Statistical analysis

The statistical software SPSS (v. 20.0 SPSS Inc., Chicago, IL, USA) was used for the analysis of the data. The difference among the treatments was computed using analysis of variance (one-way ANOVA) following Tukey's test at a significance level of 5% ($p \leq 0.05$).

RESULTS

Ethyl acetate sample of the bark of *P.chinensis* at 3000µg/disc showed maximum efficacy against *S. typhi* and formed an inhibition zone of 69.04% (ZI), while the same sample at 2000µg/disc and n-hexane extract at 3000 µg $disc^{-1}$ produced an equal zone of inhibition (38.09% ZI) against the tested bacterium as compared to control (Tab. 2). Methanol extracts produced 42.85% and 47.61% zone of inhibition against *S. typhi* at 2000 µg $disc^{-1}$ and 3000 µg $disc^{-1}$ respectively. The tested extracts were also active against *P. aeruginosa*. Among the tested sample ethyl acetate formed a maximum percent zone of inhibition (40.4% ZI) against *P. aeruginosa*. Methanol and n-hexane extracted produced the same activity at 3000µg/disc and 1000µg/disc (34% ZI at 3000 & 27.6 ZI at 1000 µg/disc).

Against *B. atrophaeus,* the methanol extract showed strong antibacterial potential than the other extracts and formed a maximum percent zone of inhibition (54.76% ZI) at 3000µg/disc. Analysis of the data also indicated that methanol extracts at 2000µg/disc and ethyl acetate at 3000µg/disc measured equal zone of inhibition (52.38% ZI). Furthermore, the n-hexane extracted sample of the bark was less effective against *B.atrophaeus.* The findings of the research study further revealed that the ethyl acetate extracts were effective than other extracts against *E. coli*, *C. freundii*, *A. tumefacien* and *R. daucus*. Ethyl acetate extract inhibited the growth of *C. freundii*, and produced 66% zone of inhibition followed by hexane extract (44% ZI) at 3000 µg/disc. The least zone of inhibition was formed by methanol extract. The n-hexane extracts at 2000 and 3000µg/disc produced the same percent zone of inhibition (44% ZI) against *C. freundii* (Tab. 2).

On the other hand ethyl acetate extracted samples produced the same activity against *A .tumefacien* and *R. daucus* (56.7% ZI) followed by methanol extract at 3000µg/disc. Furthermore, the same extracts also inhibited the growth of *E. coli* by producing a 40% zone of inhibition as compared to the positive control. Of note hexane and methanol extracts produced equal percent zone of inhibition against *E. coli* at 1000 (27.6 % ZI) and 3000µg/disc (34% ZI).

Table 2. Anti-bacterial potential of the different extracts of the bark of *Pistacia chinensis* against the bacteria.

Bacteria species	Extracts	% zone of inhibition ±STDV 1000 µg disc-1	2000 µg disc-1	3000 µg disc-1
P. aeruginosa	Methanol	27.6 ±1.6	29.7±1.1	34.1±1.9
	n-Hexane	25.5 ±1.5	27.6±1.3	34.0±1.3
	Ethyl acetate	29.7 ± 2.6	34.9± 2.1	40.1±2.2
E. coli	Methanol	27.6± 1.4	29.7 ±1.3	34 .0± 1.9
	n-Hexane	27.6 ±1.5	25.5±1.4	34.1± 1.6
	Ethyl acetate	29.7± 1.7	34.0 ±1.5	40 .1±1.2
S. typhi	Methanol	35.7± 1.9	42.8±1.6	47.6±1.6
	n-Hexane	21.4±1.5	35.7±1.7	38.1±1.5
	Ethyl acetate	38.3± 1.6	50.1±1.9	69.0±2.1
A. tumefaciens	Methanol	37.8±1.1	45.9±1.6	48.6±1.2
	n-Hexane	32.4±1.7	43.2±1.7	48.6±1.2
	Ethyl acetate	48.6±2.1	48.6±1.6	56.7±1.7
X. oryzae	Methanol	42.3±1.5	50.0±1.5	57.5±1.3
	n-Hexane	25.2±1.4	35.1±1.1	47.4±1.6
	Ethyl acetate	30.5±.9	40.1±1.3	47.5±1.9
R. daucus	Methanol	34±1.9	43.2±1.6	47.7±1.6
	n-Hexane	34±1.7	36.2±1.9	45.4±1.6
	Ethyl acetate	22.7±1.2	45.4±1.5	56.7±1.4
C. freundii i	Methanol	31±1.4	33.2±1.2	33.1±1.3
	n-Hexane	22±1.3	44.1±1.3	44.0±1.3
	Ethyl acetate	51±0.9	57.1±1.9	66.1±1.9
B. atrophaeus	Methanol	42.0±1.6	52.0±2.4	54.1±1.7
	n-Hexane	21.0±1.3	21.1±1.7	28.2±1.6
	Ethyl acetate	40.0±1.1	47.1±2.6	52.1±1.4

Concentration; 1000, 2000 and 3000 µg of the extract solution applied to the disc; STDV stands for standard deviation.

Antioxidant activity and total polyphenol content

Results for the radical scavenging activity of the tested extracts at different concentrations (25, 50, 125, and 250 µg mL^{-1}) are shown in Fig. 2. Maximum antioxidant activity was found in methanol extract compared to ethyl acetate and hexane extract (Tab. 3). In methanol extract, the percent radical scavenging activity was 79% while the hexane and ethyl acetate extract produced and 52% and 65 % respectively, at 250 µg mL^{-1}. Furthermore decreasing in the concentration of extracts decreased the % DPPH radical scavenging activity. All the extracts showed less potential to scavenge DPPH radical at 10 µg mL^{-1}. Hexane extract expressed 21% radical scavenging activity while methanol and ethyl acetate extract produced (31%) the same response to the negative radicals. On the other hand, the polyphenol assay of the different extracts revealed a significant difference in different extraction solvent systems ($p < 0.05$). The maximum concentration of total polyphenol was noted in methanol (517.25 mg g GAE^{-1}) extract followed by ethyl acetate (393.97 mg g GAE^{-1}) and n-hexane extract (378.7 mg g GAE^{-1}).

Table 3. Total polyphenol content and percent antioxidant activity in the different extracts of the bark of *Pistacia chinensis*

Extracts	Percent Antioxidant activity Mean ±SD	Total polyphenol content (mg of GAE g of $extract^{-1}$ ±sd)
Methanol extract	43.384 ± 14.8 a	518.38±1.5 a
Ethyl acetate extract	23.249 ± 2.4 b	377.48±1.7 b
Hexane extract	22.042 ± 2.7 b	383.60±1.9 c

The values in the same column represented by different letter (a-c) differ significantly at $p \leq 0.05$ using Tukey's test. GAE: Gallic acid equivalent; ±SD represent the standard error.

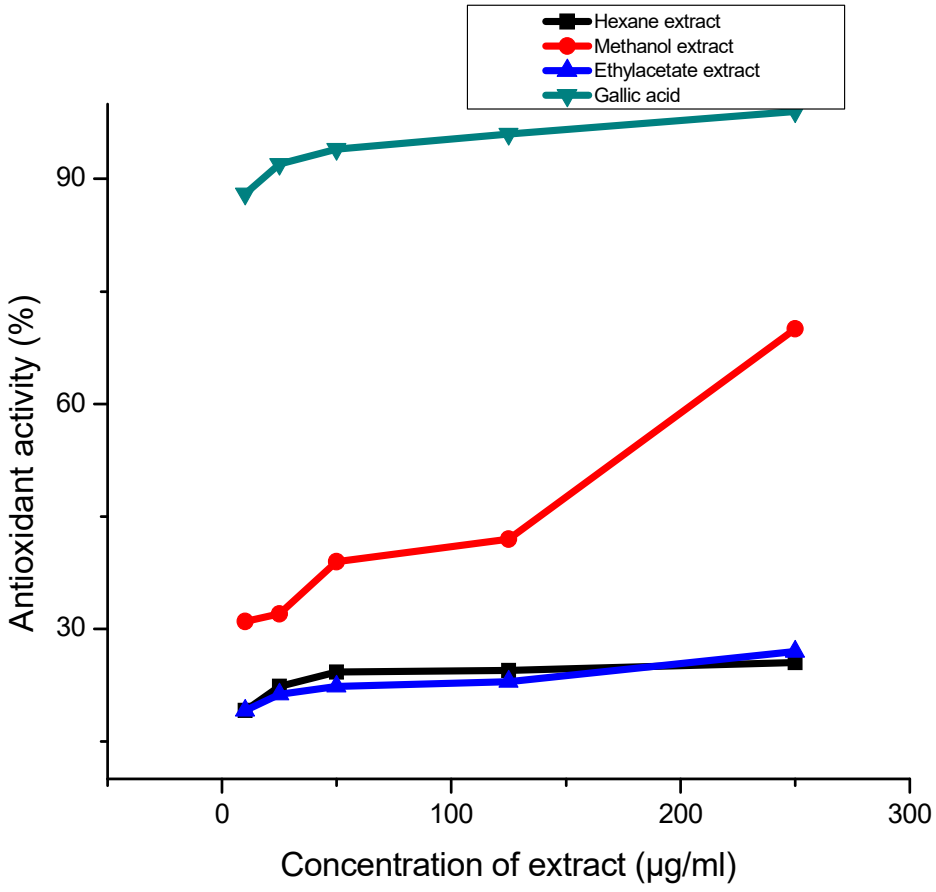

Figure 2. Antioxidant activity of different solvent extracted samples of the bark of *Pistacia chinensis*

DISCUSSION

The extraction method and the solvent used in the extraction from plant material affect the extract yield, recovery of bioactive compounds, and the bioactivities associated with the extracts (Altemimi *et al.* 2017, Wong & Kitts 2006, Trosznska *et al.* 2002). Here we have used three different extraction solvents and their effect was investigated on the antimicrobial potential, antioxidant activity, and total polyphenols content. The results of the study showed that the extracts from the barks were active against the tested bacteria. Among the tested extracts the ethyl acetate was more effective than other extracts. The ethyl acetate extract produced a maximum zone of inhibition against *C. freundii*, *S. typhi*, *E.coli* and *A. tumefaciens.* Among the tested microbes *S. typhi* and *C. freundii* were more sensitive to the ethyl acetate extract. A previous report regarding the antibacterial activity of the bark of *P. integerrima* revealed that methanol extract of bark showed strong activity against *S. typhi* and *P. aeruginosa* than ethyl acetate extract (Rahman *et al.* 2011). This variation in antibacterial activity of *P. integerrima* and *P. chinensis* against *S. typhi* indicated the difference in the structure of phyto chemical constituents of their barks and their solubility in solvents of different polarity (Uddin *et al.* 2011). The current findings were supported by the previous results which reported that the chemical nature and di electrc constant of the extraction solvent effect the antimicrobial potential of the plant material (Felhi *et al.* 2017).

On the other hand, methanol extract was more effective against *X. oryzae* and *B. atrophaeus.* High activity against *S. typhi* and *P. aeruginosa* was also found in methanol extract of the leaf of *P. chinensis* (Rashed *et al.* 2016). The strong antimicrobial activity in methanol extract may be associated with the high concentration of polyphenol in the methanol extract reported in this study. The antimicrobial potential of polyphenol has also been declared in previous studies (Shan *et al.* 2007). Furthermore, the reported compounds like terpenoids, phenolics and flavonoids in *P. chinensis* leaves (Rashed *et al.* 2016) also supported the present findings of the study. The antibacterial activity of the extracts was increasing with the increase in the concentration of the extracts. Haghgoo *et al.* (2017) also reported similar findings.

The role of negative radicals in the development of heart diseases, cataracts, and aging have been reported in recent studies (Asimi *et al.* 2013). The herbal extracts are rich in antioxidants, which help in the cure of oxidative stress-related disorders (Tan *et al.* 2018). The antioxidant potential of the extract-produced from the medicinal plant

depend on the extraction solvent and extraction methods (Khan *et al.* 2018, Anokwuru *et al.* 2011). The effect of the extraction solvent on the antioxidant potential of the extract was also observed in this study. The DPPH assay of the different solvent extracted samples revealed that methanol extract displayed strong antioxidant potential than other tested samples. Among the samples analyzed through DPPH assay, methanol extract showed high antioxidant potential than other extracts. The previous literature also reported the same findings in other species of medicinal plants (Dhawan & Gupta 2017, Chavan *et al.* 2013). Similarly, a high concentration of total polyphenol was noted in methanol extract. Polyphenols are bioactive compounds showing different pharmacological activities such as antiviral, antibacterial, anti-inflammatory, and anticancer activities (Sivaraj *et al.* 2018, Abdel-Hameed *et al.* 2012, Moyo *et al.* 2012). The current study revealed that methanol is a good solvent for the extraction of polyphenol. Similar findings were also reported in previous studies conducted on the other species of genus Pistacia (Botsaris *et al.* 2015, Jouki *et al.* 2010).

The previous research studies had reported the correlation between antioxidant potential and total polyphenol content of the extracts of medicinal plants (Yang *et al.* 2002). However, the current findings of the study did not establish the bridge between total polyphenol and antioxidant activity of the extracts. The failure of the linkage between total polyphenol and antioxidant of the extracts may be due to number of factors: the ant antioxidant potential observed may not be only due to the phenolic contents but probably due to the presence of other bioactive compounds as well as synergistic effects among them (Rahiman *et al.* 2013, Bajpai *et al.* 2005). Moreover, the Folin–Ciocalteu assay is not an absolute way for the measurement of total polyphenol due to few limitations associated with Folin–Ciocalteu reagents (Khan *et al.* 2016, Granger *et al.* 2011). The concentration and molecular geometry of phenolic compounds effect the relation between total polyphenol and antioxidant of the extract. Their antioxidant potential are associated to phenolic rings and hydroxyl groups ((Gülcin 2012, Minatel *et al.* 2017). Other factors which influence the antioxidant activity of polyphenols includes their solubility, degree of polymerization, acetylation glycosylation and interaction with other phenols (Manach *et al.* 2005). This lack of correlation between antioxidant activity and total polyphenol content is in accordance to previous reported literature (Anagnostopoulou *et al.* 2006, Nickavar *et al.* 2007, Hou *et al.* 2003). Furthermore, the non-phenolic compounds may have a role in the antioxidant potential of the bark of *P. chinensis* (Fernandes de Oliveira *et al.* 2012).

So it was found that Ethyl acetate extract is more effective than other extracts against the tested bacteria. The methanol extract showed high antioxidant activity and total polyphenol content. However, no bridge is established between the total polyphenol content and antioxidant activity. The results of the study recommended the ethyl acetate and methanol extracts for the extraction of bioactive compounds to uncover the source of the bioactivities of the extracts.

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