INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 18, No. 1, p. 15-25, 2021

RESEARCH PAPER OPEN ACCESS

Quantification of phenolic compounds of wild plants *Pulicaria gnaphalodes* and their antimicrobial activity

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Key words: Phenolic compounds, Antioxidant, Polyphenol, Extract.

http://dx.doi.org/10.12692/ijb/18.1.15-25 Article published on January 20, 2021

Abstract

The current study was used to determine the antioxidant potential of aqueous methanolic extracts of roots of *Pulicaria gnaphalodes* and quantified the bioactive compound through HPLC. The aqueous methanolic extract of roots recorded the highest total polyphenols content (TPC) 41.56 mg GAE/g of DM and total flavonoid contents (TFC) 29.58 mg QE/g of DM. Results showed that maximum DPPH free radical scavenging activity and reducing power was observed with an aqueous methanolic extract about 88% and 1.41 nm absorption respectively. Antibacterial activity of the different extracts showed that most of the bacterial strains tested were sensitive to *Pulicaria gnaphalodes* extracts.

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Introduction

The plants containing medicinal properties are due to the presence of antioxidants compounds such as phenolic, flavonoids, anthocyanins and tannins compounds (Shariatifar *et al.,* 2012). The *Pulicaria* species in Iran are commonly known as "Shabang" and "kak kosh" and are generally consumed as herbal teas, drug production and flavoring agents. The height of *Pulicaria gnaphalodes* is about 10-30cm and yellow golden flowers, which are found excessively on stony and sandy places of the Afghanistan, Western Tibet, Iran, Turkestan and Saudi Arabia (Kamkar *et al.,* 2013). These antioxidants are present in various parts of medicinal plants such as leaves, stems, roots and barks and are proved very beneficial for the treatment of various diseases. Reactive oxygen species are produced during oxidative stress and embedded with the lipid membrane. They produced free radicals in the body which cause different degenerative disorders such as cancer, diabetes, obesity, cardiovascular diseases, and inflammation, etc. Researchers have diverted their attention to the utilization of phytochemicals from fruits and vegetables to prevent oxidation.

These compounds neutralize the free radical production and prevent various diseases are generating like cancer, atherosclerosis, heart diseases and injury inflammation (Halliwell, 1997). The diseases due to the microorganism are also becoming more prevalent day by day. Therefore, the researcher is more conscious of the production of new drugs which have more potential against resistive microorganism (Mickymaray *et al.,* 2015). The current study was designed to quantify the phenolic compounds from *Pulicaria gnaphalodes* using high performance liquid chromatography and also examine the antimicrobial activities of resultant bioactive moieties.

Material and methods

The fresh plant materials were obtained from Quetta, Pakistan and further have been characterized in the Department of Botany, Government College University Faisalabad.

Extraction procedure

The *P. gnaphalodes* plant roots were selected for determining the antioxidant and antimicrobial properties. The roots were washed to remove the dust and then dried to prepare the fine powder through the grinder. The resultant powder was used to prepare the antioxidant extracts by using different solvents such as methanol and aqueous methanol. Further, solvents were shifted to orbital shaker over-night and then extracts filtered through the rotary evaporator. The concentrated samples were placed inside the freezer at 4°C for further analysis.

Preliminary phytochemical screening

The prepared aqueous and methanolic extracts were investigated for phytochemicals compounds i.e. alkaloids, tannins, terpenoids, glycosides, phenols, steroids and terpenoids according to the protocols of Chapman & Hall (1998).

Antioxidant indices

Total phenolic contents: The total phenolic contents were determined by the Folin-Ciocalteu method. Approximately 0.1ml of methanol extract and 1ml of Folin-Ciocalteau reagent were mixed in a 20ml calibrated flask. After 1minute, 5ml of sodium carbonate (20%w/v) was poured into the flask and distilled water was added until the volume reached 20ml of the end of the flask. The mixture was stored in the dark at normal temperature (25°C) for 30 minutes and then the absorbance of the solution at 760nm was detected with a UV-VIS spectrophotometer. Total phenolic contents were expressed as mg of gallic acid equivalent (Stanojević *et al.,* 2009). The value of mg GA/g was found following the formula given $y = 0.133x + 0.1089$, $R^2 = 0.9989$.

Total flavonoid contents: The total flavonoid content was found by the $AlCl_3$ method with minor modifications. About 1ml volume of 3%$AlCl_3$ was mixed with a 1ml sample solution. Then, after one hour at normal temperature (25°C), the absorbance at 430nm was determined. The appearance of the yellow color showed the existence of flavonoids in the

sample solution. The sample was examined at the 0.2mg/ml concentration range. The total flavonoids were calculated as quercetin (mg/g) through the calibration curve. The results were expressed in equivalent mg of quercetin (QE)/g extract (Turkoglu and Parlak, 2014). The absorbance was taken at 430 nm. The value of (QE)/g was found following the formula given as $y = 0.22 x + 0.039$, $R^2 = 0.9989$.

DPPH radical scavenging assay: The DPPH radical elimination test was conducted through the method described by (Williams *et al.,* 1995). The principle of DPPH was established as the reduction of DPPH due to the existence of an antioxidant hydrogen donor. The scavenging activity of the plant extract to DPPH free radicals was studied by taking various extract concentrations (such as 50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml). All of these test tubes were enclosed through the aluminum foil and kept in a dark place for 30 minutes. Then, after 30 minutes, the absorption reading was taken with a spectrophotometer at 517nm. Then the absorbance of all the different concentration sample solutions was taken. The readings were taken three times and the percentage concentration of radical scavenging was found by applying the formula:

%Antioxidant activity = [(Ac - As)/Ac] × 100
A_C blank = Control Absorbance
A_S sample = Absorbance of the test samples (Bajpai *et al.,* 2017).

Reducing power assay: The reducing capacity of *P. gnaphalodes* plant extract was studied by following the techniques of (Oyaizu, 1986). Various concentrations of extract were taken from 50-200µg/ml and dissolved in potassium ferricyanide (3.5ml, 1%water) and phosphate buffer of water (2.5ml, 0.2M, pH6.6) incubated at about (50°C) for 20 minutes. Approximately (2.5 ml) of trichloroacetic acid (10%) were mixed in the mixture and then placed in the centrifuge machine for 10 minutes. Then, after a few minutes, the solution was separated into two layers, an upper layer of solution (2.5ml) was taken and dissolved in distilled water (2.5ml) and FeCl3 (0.5ml,1%) and then the absorbance at 700nm was measured. As well as the absorbance of the mixture increases, it is shown that the reduction power also increases as reported in the previous findings (Kumaran, 2006).

High-pressure liquid chromatography (HPLC)
The quercetin was quantified through HPLC by following the guidelines of Engida *et al.* (2013). The mobile phase was used mentioned as A and B solvents A contains 1% acetic acid and B has HPLC grade methanol. Injection volume for all samples was adjusted at 60ul. The quercetin was analyzed at 280nm with 0.7 ml/min flow rate. Firstly, standard quercetin was injected after that all the sample solutions were analyzed one by one.

Antimicrobial activity
Prepared of media for bacteria: 100ml of nutrient broth medium was prepared by dissolving 2.5g/100ml distilled water, pH was adjusted 7 and was autoclaved. Nutrient agar was prepared by dissolving 12.2g/400ml distill water, pH was adjusted 7 and autoclaved.

Test organisms: The microorganisms tested were obtained from the microbiology laboratory of the Food and Biotechnology Research Center of the Council for Scientific and Industrial Research of Pakistan, in Lahore. In this study, two food-borne Gram-negative pathogens such as *Escherichia coli, Staphylococcus aureus*, and two Gram-positive subtypes of *Bacillus, Pseudomonas aeruginosa*, were used to detect antibacterial potential.

Preparation of inoculum: The bacterial strains gram-positive and gram-negative such as *Escherichia coli, Staphylococcus aureus* and two gram-positive *Bacillus subtitles*, *Pseudomonas aeruginosa* were inoculated into 10ml sterile autoclaved nutrient broth test tubes and pH was adjusted 7. These test tubes were incubated into 37°C for 24 hours. After 24 hours O.D of these four tested microorganisms and blank were observed via spectrophotometer at 600nm. The O.D of Pseudomonas was 0.360, *Bacillus subtilis*

0.595, *S. aureus* 0.336, *E. coli* 0.298 and blank 0.00 at 600nm was observed. At 600 nm 0.1 O.D is equal to 1 x 10^6 CFU/ml so *Pseudomonas aeruginosa* contained 3.6 x 10^6 CFU/ml, *Bacillus subtilis* 5.95 x10^6 CFU/ml *S. aureus* 3.36 x 10^6 CFU/ml and E.coli, 2.98 x 10^6 CFU/ml as found in previous study (Abraham and Thomas, 2012).

Preparation of stock solution and serial dilutions: The stock solution was prepared using 300mg of plant extract dissolved in 3ml of distilling water making a stock solution of 100mg/ml. Three serial dilution was prepared to obtain the concentration of 100mg/ml, 50mg/ml, and 25mg/ml.

Determination of antibacterial activity: The activity of plant extract was determined by the disk diffusion and well diffusion method.

Preparation of sterile disks: The filter no 3 of Whatman was punched into a 5 mm disc with the help of a puncture. These discs were sterilized at 121°C for 15 minutes at 15psi. These discs were immersed with the help of sterile forceps in various concentrations of 100mg/ml, 50mg/ml and 25mg/ml of plant extract after a while these discs were removed and left to air dry as reported in previous results (Mickymaray *et al.,* 2016).

Disk Diffusion method: The sterilized 20ml Agar Müller Hinton was poured into sterile Petri dishes, after solidification, 100µl of the fresh culture of *Subtitles* of *Escherichia coli, Staphylococcus aureus, Bacillus* and *pseudomonas aeruginosa* were cleaned with a buffer on the respective plates. The disks were kept on the agar plates using a sterile forceps. The plates were incubated for 24 hours at 37°C. After incubation, the diameter of the zones of inhibition formed around each disk (mm) was measured and recorded as reported (Amber *et al.,* 2017).

Hemolytic activity

The hemolytic activity of the synthetic compounds was tested by the method of (Choi *et al.,* 2011). Three milliliters of human blood cells were gently poured into a 15 ml sterile falcon tube and washed three times with 5 ml of cooled PBS by centrifuging the tubes for 5 minutes. In 2 ml Eppendorf tubes, 180 µl of red blood cell suspension was mixed with 20 µl of synthetic compounds and mixed gently.

The tubes were centrifuged for 10 minutes at 800 xg and 100 µl of the supernatant was removed and diluted with 900 µl of sterile PBS and cooled. 0.1% Triton X-100 was positive and PBS was taken as a negative control. Absorbance at 540 nm was measured in an ELISA microplate reader. Three repetitions were made for each sample.

Results and discussion

Phytochemical screening tests

The phytochemical analysis of plant extract showed that it contains several bioactive compounds (Table 1). For the phytochemical screening tests, methanolic extract of plant material was used to investigate the presence of different phytochemicals. The argument is supported by a study previously reported (Dhawan and Gupta, 2017).

Table 1. Phytochemical screening test results.

Phytochemicals	Results
Alkaloids	+
Terpenoids	+
Tannins	+
Flavonoids	+

High pressure liquid chromatography analysis of standard quercetin

The peak area of the standard was found at retention time 6.22 min about 22352387.65 µV·s which was indicated that the maximum value of standard quercetin obtained at 6.22min time range as shown in Fig. 1.

High-pressure liquid chromatography analysis of pulicaria gnaphalodes plant roots extract

The peak value of the sample was observed about 6341146.91µV·s at the retention time 6.745min minutes which was approximately the same time at which the peak value of standard was formed. The

HPLC analysis of Fig. 1 showing the existence of standard quercetin in the plant extract (*pulicaria gnaphalodes*). The major thing which was investigated through the calculation that 0.28mg/mL quercetin was present in the plant extract. Through following the same procedure standard quercetin was investigated in several other plant extracts as reported (Senhaji *et al.,* 2017). The peak area of standard quercetin was found to be 22352387.65µV·s at retention time 6.22 min.

Table 2. Antimicrobial activity of *pulicaria gnaphalodes* plant extracts at different concentrations against various bacterial strains.

Test organism	Zone of inhibition (mm)		
	25 mg/ml	50mg/ml	100mg/ml
P. arginosa	0	0	0
E. coli	2.5	0	0
S. Aureus	0	2.5	0
B. subtilis	3.5	0	0

Antioxidant activity of P. gnaphalodes plant extract

Total phenolic contents

The total phenolic contents were investigated by taking standard Gallic acid and using their already known values. The total phenolic contents were investigated from plant *P. gnaphalodes* various extracts about 40.23mg/g with methanol extract, 25.24 with aqueous extract and 41.56 with aqueous methanol extract respectively of standard Gallic acid equivalent as shown in the Fig. 3.

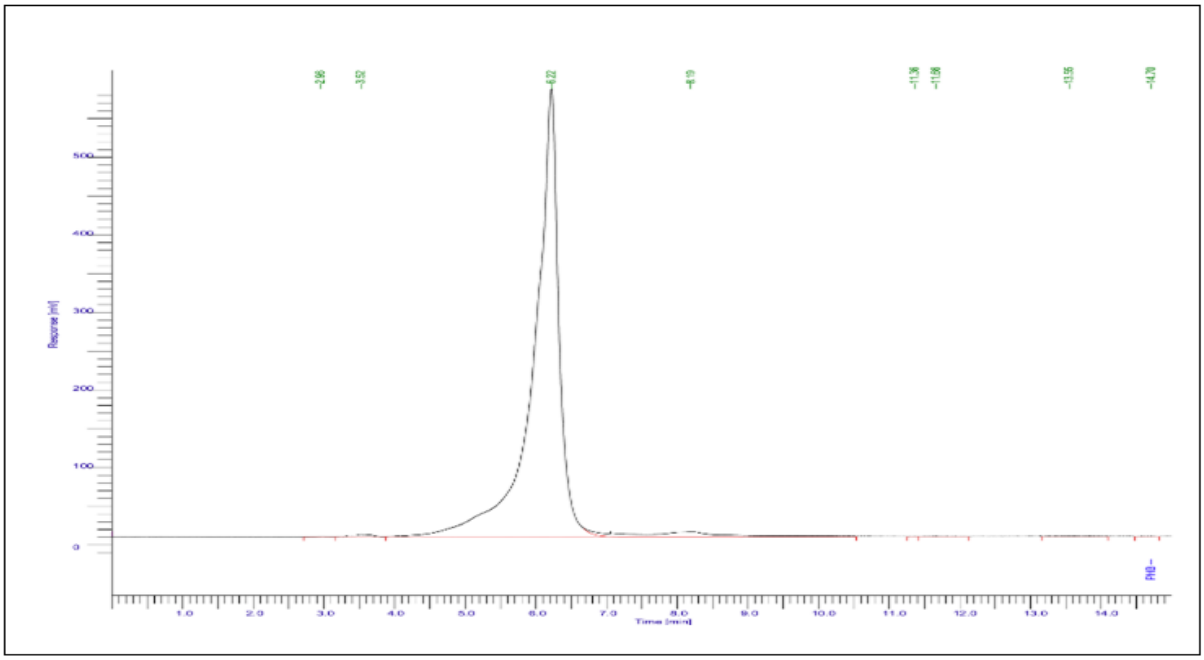

Fig. 1. High-pressure liquid chromatography analysis of standard quercetin.

Total flavonoid contents

The total flavonoids content was also determined through the comparative analysis with already known values of standard quercetin. The total flavonoid contents were found from the various extracts of *P. gnaphalodes* to be 28.43mg/g, 19.90mg/g and 29.58mg/g for methanol, aqueous and aqueous methanol extract respectively of standard quercetin equivalent as shown in the Fig. 4.

DPPH Free radical scavenging assay

The maximum free radical elimination activity was

exposed with an aqueous methanolic extract about 88% and then 87.03% with methanol extract as shown in Fig. 5. Aqueous methanol and methanol extract provides the highest free radical elimination activity due to the most flavonoids and phenolics compounds present in the methanol extract in comparison with the aqueous extract. Therefore, the methanol extract shows a high antioxidant activity (Sharma and Vig, 2013).

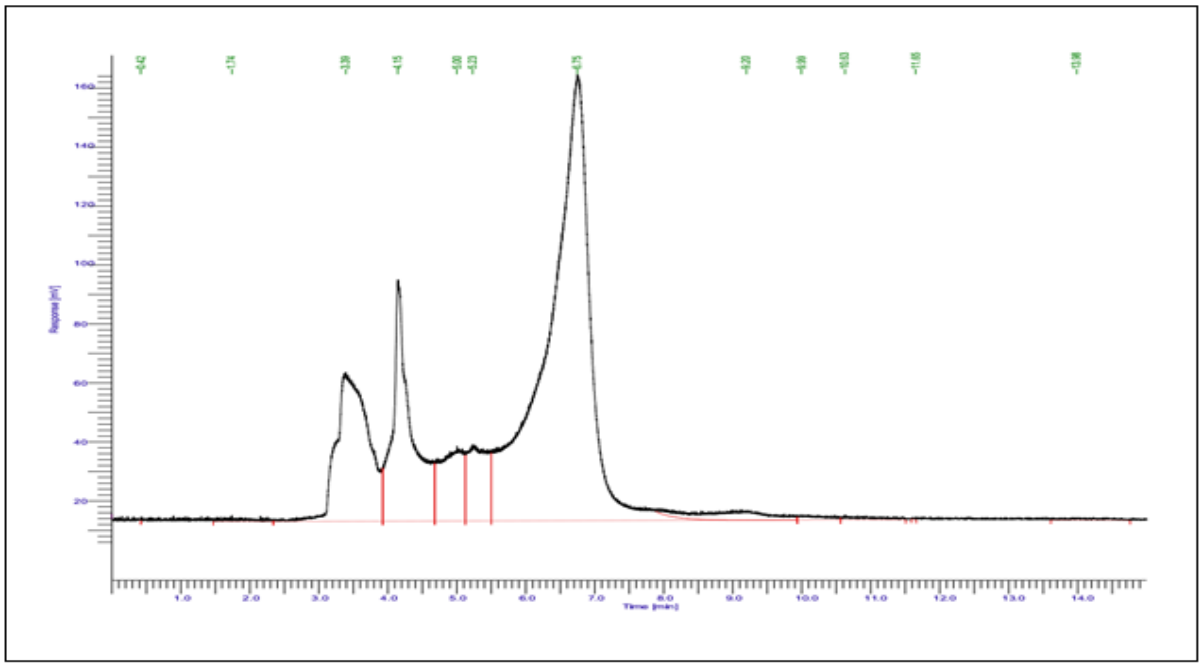

Fig. 2. High-pressure liquid chromatography (HPLC) analysis of sample *P. gnaphalodes* roots extract.

By increasing the concentration of the extract, the activity of eliminating free radicals of the extract has been increased. Maximum activity was observed at the concentration of 200µg/ml extract. The aqueous solvent scavenging activity was approximately 80.01%.

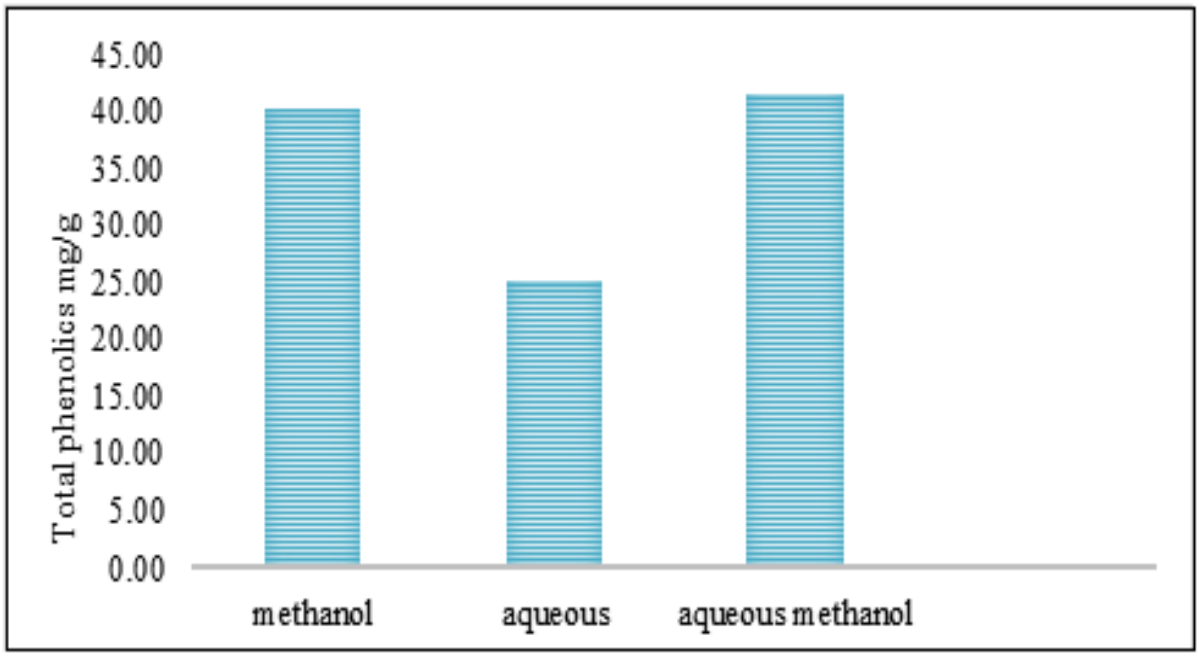

Fig. 3. Total phenolic contents of the plant extracts**.**

The analysis indicated that the aqueous extract provides a minimum scavenging activity of free radicals concerning the methanol extract which provides 87% free radical scavenging activity as reported (Nandhakumar annd Indumathi, 2013) in their research work.

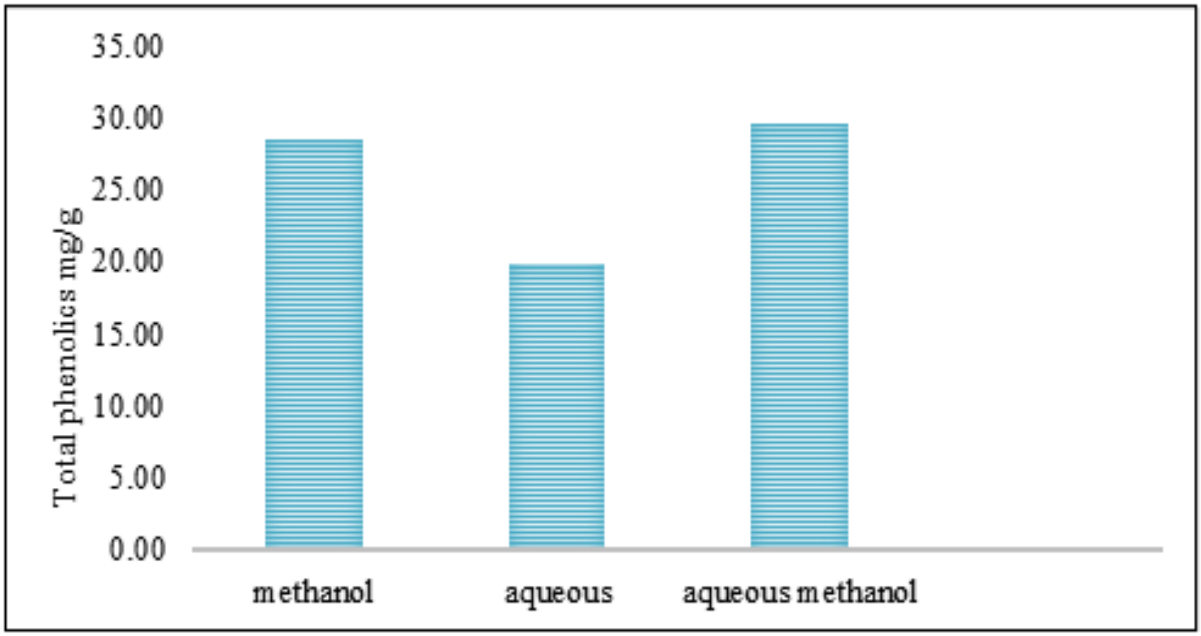

Fig. 4. Total flavonoid contents of the plant extracts**.**

The scavenging activity of plant extract to DPPH free radicals was determined by taking various concentrations of the extracts as shown in Fig. 5. By increasing the concentration of the sample the scavenging activity was also gradually increased. The scavenging activity of free radicals of DPPH has been studied for those samples that have been extracted through the aqueous, methanol and aqueous methanol solvents. The scavenging activity of free radicals of the aqueous methanol extract was approximately equal to the methanol extract which provides about 88% of the free radical scavenging activity as reported (Boeing *et al.*, 2014).

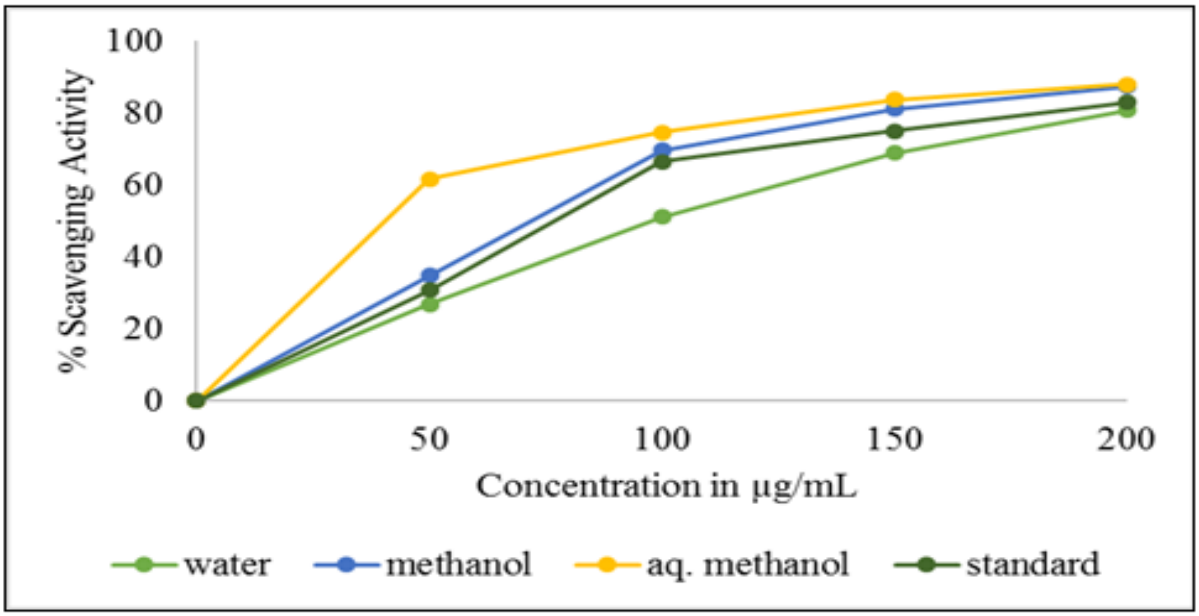

Fig. 5. DPPH Free radical scavenging assay.

The Butyl hydroxyl toluene (BHT) was taken as a reference compound for the accurate measuring of DPPH free radical scavenging results by plants bioactive compounds. The BHT absorbance was analyzed by taking a various concentration of BHT. The graph of BHT was showing that increasing the concentration of BHT absorbance value also increases gradually. In the case of methanol, extract absorbance was less than BHT but in the case of aqueous extract, the absorbance was more than the standard. The comparative analysis of standard BHT with free radical scavenging results was also reported (Pavithra annd Vadivukkarasi, 2015).

Reducing power determination of plant extracts

The reducing power of the plant extract *P. gnaphalodes* was determined using different solvent extracts such as aqueous, methanol and the mixture of water and methanol were taken to determine the variation of the plant extract reduction power through extraction with various combinations of solvents. At the concentration of 200µg/ml, the absorption value was found to be 1.20nm, as shown in Fig. 6. The plant material that was extracted with methanol gives higher absorbance value than the aqueous extract. The highest absorption value indicated that the reducing power of the methanol extract was higher than the aqueous extract as reported in the previous findings (Ahmed *et al.,* 2015). The reducing power of the aqueous extract of the plant has been determined at various concentrations of extracts. Through the analysis, it was found that by increasing the concentration, the absorbance value also increases at the concentration of 50µg/ml of extract the absorbance value was found 0.4nm and about 1nm of absorbance value at 200µg/ml of extract concentration. The reduction capacity of various plant extracts has been examined. The reducing power of the aqueous extract showed less activity than the methanol extract (Benslama and Harrar, 2016).

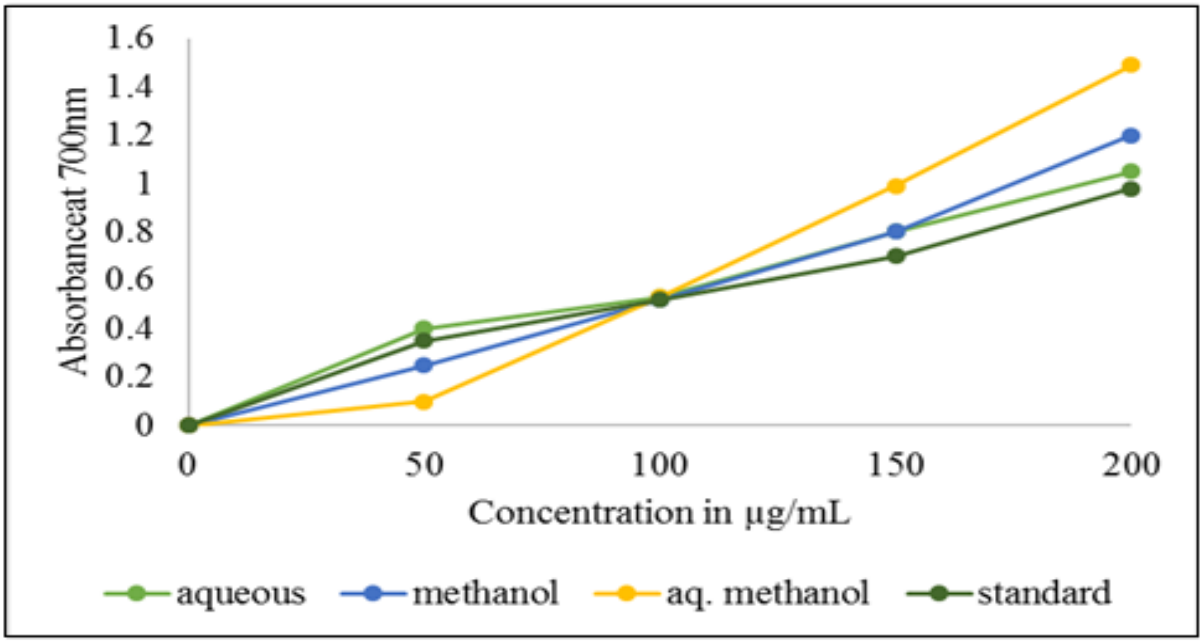

Fig. 6. Reducing power determination of plant extracts.

The reducing power of *pulicaria gnaphalodes* plant extracts was determined at various concentrations. The plant material that was extracted with aqueous methanol provides an absorption value of about 1.41nm which is superior to that of methanol and aqueous solvent extracts. It has been shown that the highest value of the reducing power of the plant material was found with the combination of these solvents as reported (Sultana *et al.,* 2009).

Antimicrobial activity of pulicaria gnaphalodes plant extract

Disc diffusion

The *pulicaria gnaphalodes* show a very weak

antibacterial activity (Table 2). The antibacterial activity is shown by extract against *S.aureus, E.coli and Bacilitus* strains. The result supports the previous findings that *pulicaria* species have very weak antibacterial potential as reported by (Asghari *et al.,* 2014).

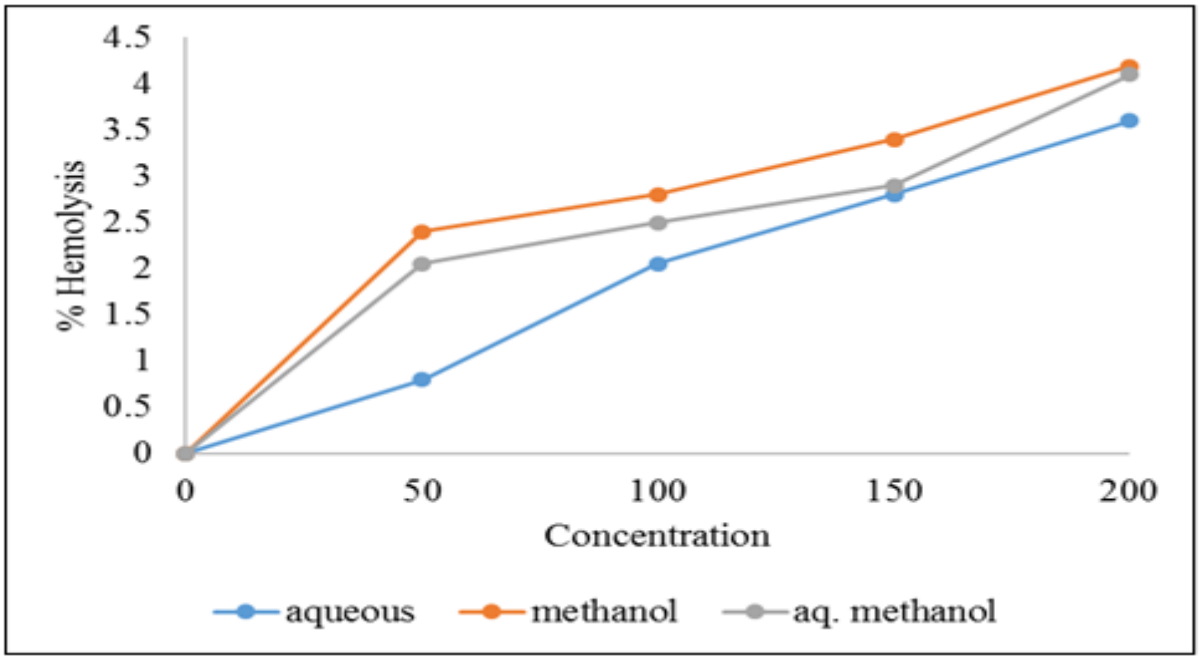

Fig. 7. Hemolytic activity of red blood cells.

Hemolytic assay

Cytotoxicity was studied by examining hemolytic activity against human red blood cells (Fig. 7). (RBC) using Triton X-100 as a positive control. The percentage of lysis evaluated by comparing the sample absorbance and Triton X-100. The positive control showed lysis of approximately 100%, while phosphate-buffered saline (PBS) showed no red blood cell lysis (Riaz *et al.,* 2012). When the extract fractions were made into interaction with control the % lysis of RBCs was observed. The mechanical stability of the red blood cell membrane is a good indicator for evaluating in vitro the effects of various substances tested in the detection of cytotoxicity (Sharma and Sharma, 2001). The % lysis for all fractions at designated concentrations were less than 5%. The aqueous extract showed least % lysis when compared with other fractions.

Conclusion

Natural products have been recognized and used to produce medicines worldwide. These herbal medicines are prepared by the phytochemicals that have been utilizing to treat the human maladies. Antioxidants are present in higher concentrations in different parts of these plants which further work as chemo-preventive and antimicrobial agents. In conclusion, further investigations on this plant can open new horizons in the field of medicine.

Conflict of Interest

The author declares no conflict of interest.

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