

***Curcuma longa* (Turmeric): An auspicious spice for antibacterial, phytochemical and antioxidant activities**

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Abstract: Turmeric, a recognized spice, is known for miscellaneous health benefits in addition to culinary uses. In this study, *in vitro* evaluation of turmeric ethanol, methanol and aqueous extracts were mediated by disc diffusion, agar well method and phytochemical analysis. Purification of curcumin from turmeric was assisted by silica gel, TLC and HPLC for evaluation of its antioxidant and DNA protection activity. The sensitivity of alcoholic extracts against bacterial species differed, yet *Staphylococcus aureus* subsp. *Aureus* and *Bacillus subtilis* both exhibited pronounced inhibition in disc diffusion and agar well method respectively. Overall, the crude ethanol extract of turmeric has an enhanced inhibitory effect on the growth of different bacterial species with a mean of 9.4 ± 1.00 mm compared to 8.8 ± 0.58 mm in case of crude methanol extract. Phytochemical analysis confirmed the presence of carbohydrates, flavonoids, coumarins, steroids, saponins, tannins and phenols. Purification of curcumin through HPLC gave the main peak with 55% of acetonitrile at a retention time of 61- 65 minutes. Lower concentration of purified curcumin has protective effects on human DNA but increased concentrations instigate damaging effects. Its percentage scavenging ability was highest (91.84%) at 45 μg and per unit increase in the concentration prompted 6 units increase in percentage inhibition with a linear regression, $R^2 = 0.914$. All these traits boost its significance in herbal medicine with varied antimicrobial and pharmacological activities.

Keywords: Turmeric, curcumin, antibacterial, health benefits.

INTRODUCTION

Throughout time, the use of plants and plant-derived products have and continue to offer a plethora of health benefits. In addition to rich culinary uses, spices in particular, contribute striking health benefits (Newman *et al.*, 2003). Some herbs and spices are known to exhibit anti-cancerous and antioxidant activities (Tapsell *et al.*, 2006). *Curcuma longa* (Turmeric), a well-known spice, is one of 80 species within the *Zingiberaceae* family. Many of its species are known to exhibit anti-inflammatory effects and other notable health benefits owing to curcumin and beta-carotene. Application to disease, including a range of human cancers, has shown to elicit significant effects (Hewlings and Kalman, 2017). Hence, turmeric is currently a subject of great interest due to its profits in the medical field (Sharma *et al.*, 2013; Nelson *et al.*, 2017).

Turmeric plant growth and survival demands 20°C to 30°C temperature and a specific amount of the rainfall. The rhizomes are typically dried and crushed to a fine powdered form used as seasoning. Its rhizomes contain 60% to 70% carbohydrates, 6% to 8% proteins, 3% to 7% essential oils, 5% to 10% fixed oils, 2% to 7% fiber, 3% to 7% minerals and mainly 2% to 6% curcuminoids (Chattopadhyay *et al.*, 2004). Turmeric exhibits good antibacterial potential against different negative and

positive strains including *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* in the form of crude extracts. These turmeric extracts are also inhibitive for fungi such as *Mucor* species and *Rhizopus stolonifera* (Pundir and Jain, 2010). Curcumin, an important component of turmeric, was also found to possess antimicrobial activity against different strains of *Streptococcus pneumonia* including *penicillin* resistant. By analysing the zones of inhibition and minimum inhibitory concentration, it was concluded that different derivatives of curcumin exhibit great antimicrobial potential notably against *penicillin* resistant strains. As a result, it was unambiguous that these derivatives could be prescribed for the treatment of *penicillin* resistant strains (Li *et al.*, 2016).

The discovery of curcumin relates to when Vogel and Pelletier isolated the yellow colored pigment from rhizomes of *C.longa*. Since then, it has been identified as an active curcuminoid present in turmeric (Molobedzka *et al.*, 1910). It is known to be a good antioxidant, anti-inflammatory agent, anti-carcinogenic agent, anti-mutagenic agent, and anti-coagulant, widely used for combating a range of health issues. These diverse, protective functions are owing to antioxidant molecules that protect DNA and tissues from oxidative damage. Curcumin is a polyphenol of hydrophobic nature with very low water-solubility, low stability, higher metabolism and very low absorption rate. Together, these properties decrease the bioavailability of curcumin,

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limiting its clinical use. Nevertheless, drug delivery systems have been modified to enhance the biosorption and permeability of curcumin and to decrease its presystemic degradation in order to overcome this limitation (Bhattacharyya *et al.*, 2014). Recently, amorphization has been recognised as a valuable technique for solubilisation of low water soluble drugs like curcumin as reported by Kimura *et al.* (2018).

In this study, we have evaluated the antimicrobial activity of the crude ethanol and methanol extracts of turmeric against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* subsp. *Aureus*, *Bacillus thuringiensis* and type strain of *Bacillus endophyticus*. The diameters of the inhibitory zones produced by these extracts were measured in millimetres (mm) and subject to statistical analysis. Phytochemical analysis of the crude turmeric aqueous extract was used to identify key secondary metabolites. Furthermore, antioxidant assays were used to assess the bioactivity of purified curcumin.

MATERIALS AND METHODS

Sample collection and bacterial strains

Dr. Najam us Sahar, a taxonomist, identified the dried rhizomes of turmeric purchased from a local market of Lahore, at University of the Punjab, Lahore, Pakistan. The bacterial strains *Escherichia coli* (ATCC® 8739™), *Bacillus subtilis* (ATCC® 10774™), *Staphylococcus aureus* subsp. *Aureus* (ATCC® 25923™), *Bacillus thuringiensis* (ATCC® 13367™) and type strain of *Bacillus endophyticus*: DSM 13796 were provided by the Institute of Biochemistry and Biotechnology, University of the Punjab in the form of glycerol stocks. They were refreshed by streaking them on LB (Luria Bertani) agar plates and the dried rhizomes were crushed to fine powder for further experimentation.

Extracts preparation and sub-culturing

Turmeric extracts were prepared by dissolving 25 g of powdered rhizome in 95% ethanol or methanol and kept at 4°C for 24 hours. The filtrate was concentrated and kept at 4°C for further use. Antibacterial activities of the crude turmeric extracts were assessed by inoculating five selected bacterial species into the autoclaved (15 psi and 120°C) LB broth in separate tubes. The tubes were then kept at 37°C overnight to provide suitable growth conditions for the bacteria.

Antibacterial assays

For evaluating the antibacterial activity of the crude extracts, 100µl of each strain was poured on the solidified agar plates (0.5% yeast extract, 1% tryptone, 1% NaCl, 2% agar and pH 7.4). Paper discs (4 mm wide), impregnated in the crude ethanol and methanol extracts were placed on top. The plates were set aside for pre-diffusion for 10 minutes and then incubated at 37°C

overnight. Solvents ethanol and methanol were used as negative control and Ampicillin (1 mg/ml) was used as a positive control.

For agar well method, bacterial lawns were hollowed out by 4 mm borer and filled with 30µl of each extract, kept undisturbed for 10 minutes for the purpose of pre-diffusion, followed by incubation at 37°C overnight. The diameters of the zones of inhibition were measured and statistical analysis was done by GraphPad Prism 7.03.

Phytochemical analysis

Aqueous extract was prepared by dissolving 100g turmeric powder in 250 ml distilled water for 2 days with continuous mixing. Filtrate was subjected to further phytochemicals analysis (Sawant and Godghate, 2013). Ethanol extract of turmeric (as prepared above) was used for the phytochemical analysis of steroids.

Purification of curcumin from turmeric

For extraction of curcumin from turmeric, 20g of powdered turmeric was dissolved in 200 ml of Acetone and subjected to continuous stirring at 4°C for 48 hours. Filtrate was lyophilized (-70°C) and stored at -20°C for further purification (Kulkarni *et al.*, 2012).

Silica gel column chromatography

In the first step, Silica gel column chromatography was employed in order to access purified curcumin. Silica gel 60 and chloroform: methanol (95:5) solution was used to pack the column (34 cm×1.5cm) and washed with double distilled water. Concentrated mass (2 gm) dissolved in acetone (1 ml) was subjected to the column along with elution buffer (chloroform: methanol; 95:5). A total of 50 fractions were taken with a flow rate of 1.5 ml/min.

Thin layer chromatography

Standard curcumin (crystalline) was purchased from BDH Chemicals Ltd England (Product no. 20031) as a reference. Reference and lyophilized test sample were dissolved in 100µl of acetone to make a final concentration of 1 mg/ml. Separate (20 µl) spots for each fraction were applied on TLC plate and dried samples were allowed to run up to a length of 13 cm in elution buffer (Chloroform: Methanol) placed in chromatographic tank. The plates were dried and the spots were analysed in comparison to the standard spot, to detect the presence and concentration of curcumin. The R_f values were calculated using the following formula:

$R_f \text{ value} = \frac{\text{Distance travelled by the spot}}{\text{Distance travelled by the solvent front}}$

Reverse phase HPLC

Reverse Phase HPLC (SYKAM, Germany GmbH) was used for further purification of curcumin at 420 nm using Biobasic C18 analytical column (250×4.6 mm) in contrast to standard (Curcumin). The sample was run using

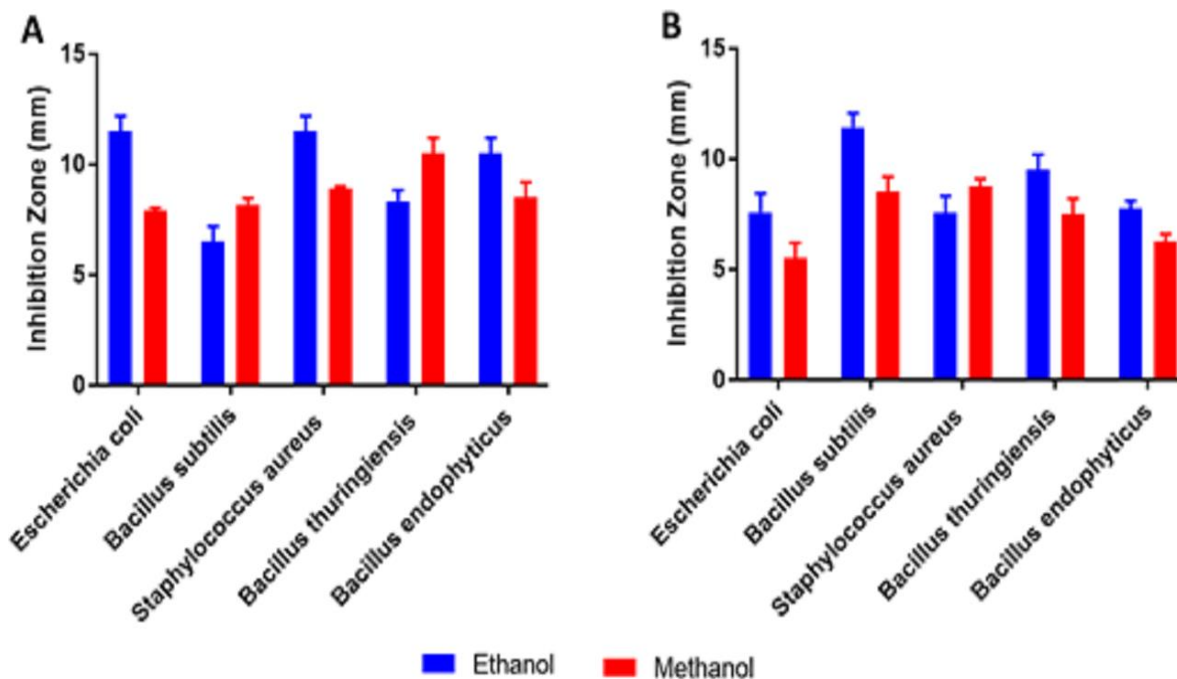


Fig. 1: Graphs representing antibacterial activity of alcoholic extract of Turmeric against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* subsp. *Aureus*, *Bacillus thuringiensis* and type strain of *Bacillus endophyticus*; (A) In disc diffusion method, (B) In agar well method.

solvent A (0.1% TFA in water) gradient for 5 minutes, followed by varied gradients of solvent B (99.9% acetonitrile in 0.1% TFA) for 100 minutes (1% ramp per minute).

Quantifying the antioxidant activity

Different concentrations of purified curcumin (15-45 μg) containing 0.5 ml of 0.1 mM (0.0394 gm of DPPH[•] in 1000 ml of water) DPPH were taken in order to assess their antioxidant activity (Borra *et al.*, 2013) from 1 mg/ml stock solution. After half an hour incubation in dark, absorbance of sample and control (containing ethanol and DPPH[•]) was measured at 517 nm. Ascorbic acid samples were run in parallel as standard. The percentage oxygen radical scavenging effect of the spice was measured by the following formula:

$$\text{Percentage DPPH}^{\bullet} \text{ scavenging effect} = (1 - A_s/A_c) \times 100$$

Where A_s is the absorbance of each sample and A_c is the absorbance of control at 517 nm. The value of the linear regression and other statistical analysis of the results were interpreted through.

DNA protection assay

Protective effect of curcumin on H_2O_2 induced DNA damage was analysed through the method of Golla and Bhimathati, 2014; with some modifications. We used normal human DNA extracted by salting out method from whole blood (Miller *et al.*, 1988). Human DNA (25 μl) was mixed with 200 μl of 20 mM of Potassium Phosphate buffer (KH_2PO_4) having pH 7.4, 30 μl of 150 mM of NaCl and final volume was made up to 1 ml with the help

of autoclaved water. Each sample was treated with 2 μl of 1 mM H_2O_2 , acetone, potassium phosphate buffer and 4 μl of bromophenol blue dye (0.5% bromophenol blue, 50% glycerol in water). Samples were incubated at 37°C for 30 minutes followed by incubation on ice for 10 minutes to stop H_2O_2 activity and analysed on 1% agarose.

RESULTS

In the present study, the antimicrobial potential of turmeric was confirmed by using disc diffusion and agar well method against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* subsp. *Aureus*, *Bacillus thuringiensis* and type strain of *Bacillus endophyticus*. In case of disc diffusion method, crude ethanol extract of turmeric has shown significant results against *Escherichia coli* (12mm), *Staphylococcus aureus* subsp. *Aureus* (11 mm) and type strain of *Bacillus endophyticus* (10 mm) in comparison to *Bacillus thuringiensis* (8 mm) and *Bacillus subtilis* (6 mm) as shown in the fig. 1A. The crude methanol extract of turmeric has shown noticeable inhibitory effect against all bacterial species; *Bacillus thuringiensis* (10mm), *Staphylococcus aureus* (9 mm), *Bacillus endophyticus* (8 mm), *Bacillus subtilis* (7 mm) and *Escherichia coli* (7.6 mm). Hence, inhibition of *Bacillus thuringiensis* and *Bacillus subtilis* was more pronounced by methanol extract of turmeric, whereas, inhibition of *Staphylococcus aureus* subsp. *Aureus*, *Bacillus endophyticus* and *Escherichia coli* was evident by ethanol extract.

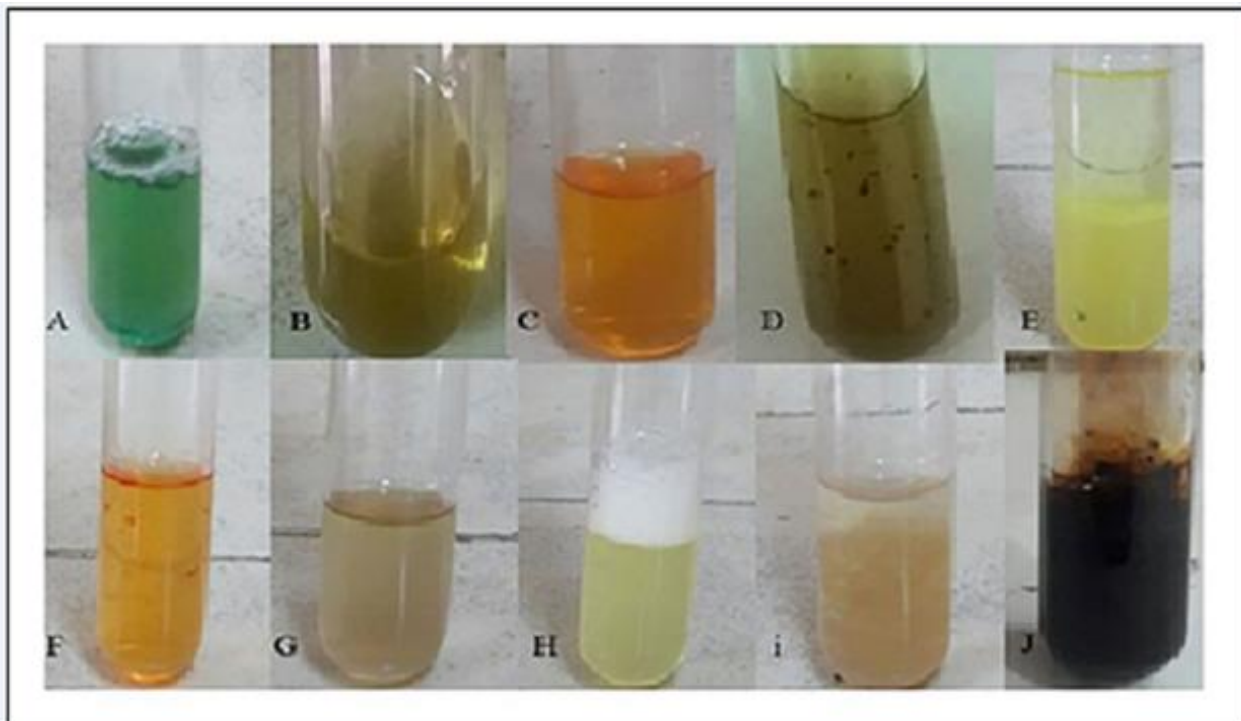


Fig. 2: Phytochemical Analysis of Turmeric; (A) The presence of green colour confirmed the positive test for carbohydrates in aqueous extract of turmeric, (B) The absence of yellow colour confirmed the negative test for proteins in aqueous extract of turmeric, (C) The presence of intense yellow colour confirmed the positive test for flavonoids in aqueous extract of turmeric, (D) The absence of blue violet colour confirmed the negative test for anthocyanin's in aqueous extract of turmeric, (E) The absence of upper red layer confirmed the negative test for leuco-anthocyanins in aqueous extract of turmeric. (F) The presence of yellow colour confirmed the positive test for coumarins in aqueous extract of turmeric, (G) The presence of upper red layer and a lower greenish layer confirmed the positive test for steroids in aqueous extract of turmeric, (H) The presence of persistent foam confirmed the positive test for saponins in aqueous extract of turmeric, (I) The occurrence of yellow precipitates confirmed the positive test for tannins in aqueous extract of turmeric, (J) The presence of blackish colour confirmed the positive test for phenols in aqueous extract of turmeric.

In case of agar well diffusion method (fig. 1B) *Bacillus subtilis* (11 mm) was more susceptible to inhibition by crude ethanol extract, while, *Bacillus thuringiensis* (10 mm) and *Bacillus endophyticus* (8 mm) were less susceptible. However, the inhibitory effect was similar against *Escherichia coli* (7 mm) and *Staphylococcus aureus* (7 mm). In contrast to crude ethanol extract, the crude methanol extract of turmeric was most effective to inhibit the growth of *Staphylococcus aureus* (9 mm) and *Bacillus subtilis* (9 mm) followed by *Bacillus thuringiensis* (7 mm), *Bacillus endophyticus* (6 mm) and *Escherichia coli* (5 mm).

The statistical analysis of the antibacterial activity of the ethanol extract has shown that in the disc diffusion method, the mean diameter of the zone of inhibition was 9.4 ± 1.00 mm while, in the agar well method the mean diameter was 8.2 ± 0.5 mm. On the other hand, the mean diameter of zone of inhibition was 8.8 ± 0.58 mm and 7.4 ± 1.1 mm in case of the disc diffusion method and the agar well method respectively. The zone of inhibition formed by the absolute ethanol and the absolute methanol

as negative controls were totally negligible compared to the zone of inhibition formed by the crude ethanol and methanol extract of turmeric. Hence, the zone of inhibition formed by the crude extracts was solely due to the antibacterial potential of turmeric and not because of the solvents used.

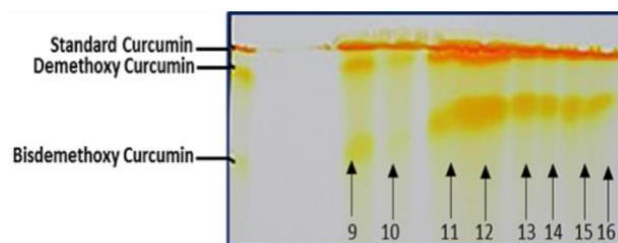


Fig. 3: TLC of turmeric disclosed that fractions 9, 10, 11, 12 and 13 had the highest concentration of curcumin.

Phytochemical analysis of the crude aqueous extract confirmed the presence of carbohydrates, flavonoids, coumarins, phenols, saponins, tannins and steroids, illustrating turmeric as a spice with diverse benefits (fig. 2). Purification of curcumin from turmeric, as following

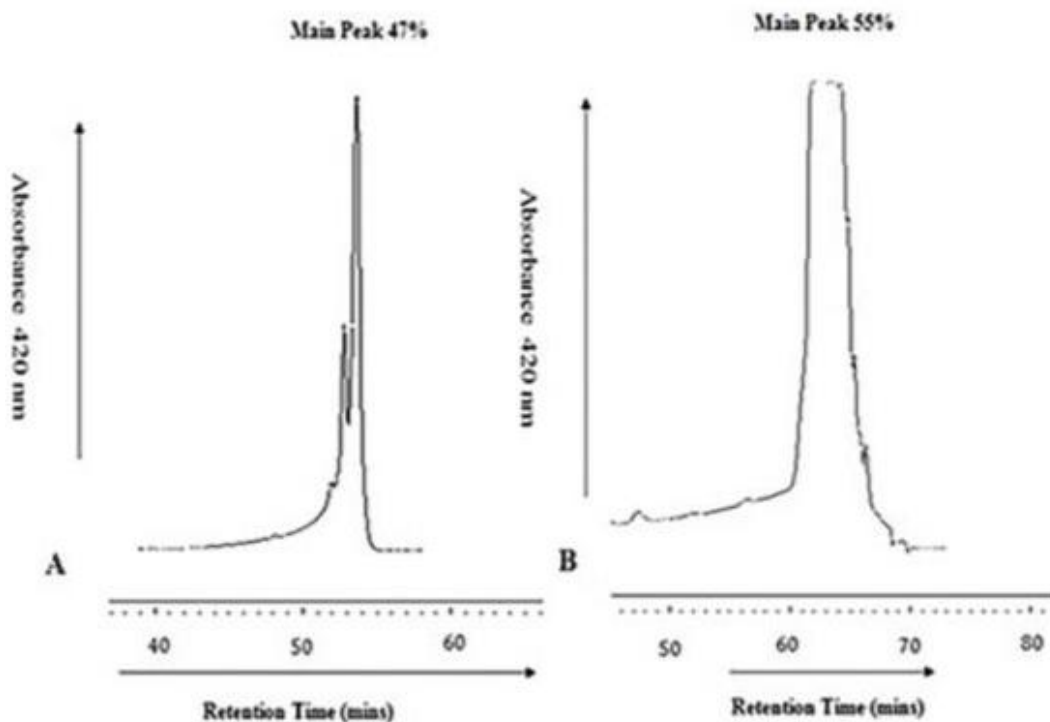


Fig. 4: Reverse Phase HPLC of Curcumin; (A) Elution at 47% acetonitrile/0.1% TFA (B) Elution at 55% acetonitrile/0.1% TFA.

the procedure of Kulkarni *et al.* (2012), confirmed the separation of curcumin along with some of its analogues like Demethoxycurcumin and Bisdemethoxycurcumin (fig. 3). A total of 50 fractions were taken with a flow rate of 2 ml/min from Silica gel 60 column chromatography. Collected fractions were subjected to thin layer chromatography in order to visualize the desired component. From the results of thin layer chromatography, Rf values of curcumin were 0.78, 0.79, 0.80, 0.80, 0.79; Demethoxycurcumin were 0.74, 0.77, 0.78, 0.78, 0.76 and Bisdemethoxycurcumin were 0.57, 0.62, 0.64, 0.65, 0.65 for the fraction 9, 10, 11, 12 and 13 respectively (fig. 3). The concentration of the curcumin was found to be greatest in the fractions ranging from 9 to 13 while the concentration decreases as the number of fraction increases. Further purification of the compound through the Reverse Phase HPLC gave the main peak of the sample eluting with 55% of the solvent (acetonitrile) at 420 nm with a retention time between 61 to 65 minutes as shown in fig. 4.

The antioxidant activity of curcumin and its potential to scavenge the reactive oxygen species (ROS) was estimated by using DPPH as a source of free radical and evaluated the percentage scavenging ability of curcumin as percentage inhibition of free radical production. In this experiment ascorbic acid was taken as a standard. The percentage inhibition (%) of curcumin was 54.88, 61.16, 70.84, 79.16, 85.88, 89.16 and 91.84 at 15, 20, 25, 30, 35, 40 and 45 μg concentration of curcumin at 517 nm (fig. 5). In DNA protection assay, 5 μl of curcumin was

effective to renature the damaged DNA, whereas increased concentrations; 10 μl of curcumin showed a relative weak potential and 15 μl had negligible protection effects, hence had detrimental effect (fig. 6).

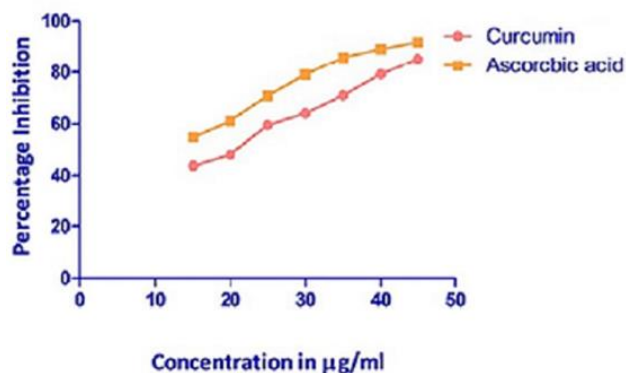


Fig. 5: Percentage inhibition potential of curcumin and ascorbic acid to scavenge DPPH \cdot radical.

DISCUSSION

Curcuma longa (Turmeric) has been explored extensively and well acknowledged for its therapeutic efficacy in Pakistan. Curcumin from turmeric was renowned for its antibacterial activity through disruption of ion channels across the bacterial cell wall. A concentration of 20 $\mu\text{g/ml}$ to 90 $\mu\text{g/ml}$ has been proven operative against various gram positive and gram negative bacterial species (Aly and Gungumjee, 2011). Turmeric has reported to have a 92.3% antioxidant activity, depictive of its protective

attributes and potential to reduce the risk for chronic diseases like cardiovascular and cancer (He *et al.*, 2015)

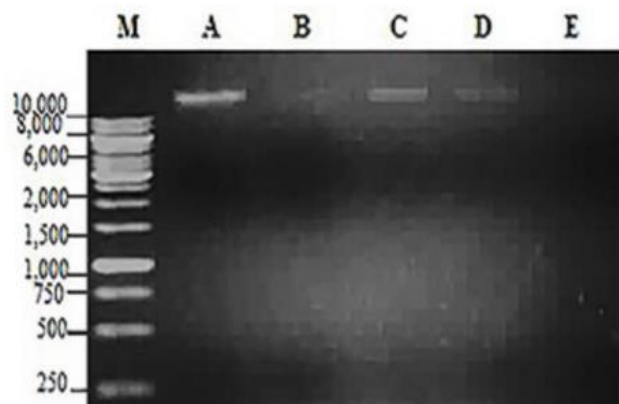


Fig. 6: DNA protection assay showing effect of different concentrations of curcumin on the damaged DNA; M 1kb Molecular marker, (A) Negative control (Normal DNA without treatment), (B) Positive control (DNA Damaged by H₂O₂), (C) DNA damage response (treated with 5 µl of curcumin), (D) DNA damage response (treated with 10 µl of curcumin) (E) DNA damage response (treated with 15 µl of curcumin).

In the present study, the antimicrobial potential of turmeric was confirmed by using the disc diffusion and the agar well method, against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus subsp. Aureus*, *Bacillus thuringiensis* and type strain of *Bacillus endophyticus*. The above mentioned strains have shown different percentage of sensitivity towards ethanol and methanol extract of turmeric (fig. 1). The antibacterial effect of crude extracts also varies in terms of methods utilised for their testing. The zone of inhibition of *Escherichia coli* against ethanol extract was 12 mm and 7 mm in case of the disc diffusion and the agar well method respectively. While, in a study conducted by Pundir and Jain (2010), the ethanol extract of turmeric inhibited the growth of *Escherichia coli* by giving a zone of inhibition of 24 mm in the agar well method but with a higher concentration of extract than used in our study. In current study, the methanol extract has shown inhibitory zones of 8 mm and 5 mm against *Escherichia coli* in the disc diffusion and the agar well method respectively. These results were contrary to those reported by Pundir and Jain (2010), in which the methanol extract produced a 28 mm zone against *Escherichia coli* in the agar well method. The difference in the inhibitory zones formed in the two studies was because of the difference in the amount of extract they used, prior used 30 µl of the extract while the later used 100 µl of extract. In another study, conducted by Mukhtar *et al.*, (2012), reported that the zone of inhibition formed by ethanol extract of turmeric, in the disc diffusion method was 9 mm against *Escherichia coli*.

In the current study, the inhibitory zone of turmeric ethanol extract against *Staphylococcus aureus subsp.* was

measured to be 6 mm and 11 mm using the disc diffusion and the agar well method while, the zone of inhibition formed by turmeric methanol extract against *Staphylococcus aureus* was 8 mm and 9 mm respectively. Kumar *et al.*, (2010) showed opposing results in their study, where the ethanol extract showed 20 mm zone against *Staphylococcus aureus* by agar well method. In another study, ethanol and methanol extract showed 20 mm and 15 mm zone of inhibition respectively, against *Staphylococcus aureus* by employing the disc diffusion method (Gupta *et al.*, 2015).

In the present research work, the zone of inhibition against *Bacillus subtilis* were measured to be 11 mm and 7 mm by the disc diffusion and the agar well methods respectively, using ethanol extract. These results were supported by Mukhtar and Ghorri (2012), where zone formed by ethanol extract in disc diffusion method was estimated to be 12 mm against *Bacillus subtilis* and 14 mm using agar well method. In the current work, we observed an inhibitory zone of 9 mm against *Bacillus subtilis* using methanol extract in both methods. Kumar *et al.*, (2010), used 100 µl methanol extract against *Bacillus subtilis* and has shown a 14 mm zone of inhibition using the agar well method.

Phytochemical analysis confirmed the presence of carbohydrates, flavonoids, coumarins, phenols, saponins, tannins and steroids, illustrating turmeric as a spice with diverse benefits (fig. 2). In this study, ethanol was used as a means to solubilize the dried steroid from turmeric because steroids have limited aqueous solubility as confirmed by the study conducted by Deb *et al.*, (2013) where they found no steroids present in the crude aqueous extract of turmeric despite the presence of all other components. In the present work, purification of an important component, Curcumin (fig. 3), along with its analogues like Demethoxycurcumin and Bisdemthoxycurcumin were in accordance with the study done by Kulkarni *et al.*, (2012). Further purification of curcumin through Reverse Phase HPLC (fig. 4) was in accordance to a study conducted by Nabati *et al.*, (2014) where they applied the same protocol at 425 nm with acetonitrile: acetic acid, 52:48 as a solvent and peaks for the sample and standard were eluted at a retention time of 15.5 to 16.5 minutes in a total reaction of 25 minutes.

A study was also conducted to evaluate the antioxidant activity of curcumin and finding out its potential to scavenge the reactive oxygen species (ROS). The result of this experiment was compared to the study by Borra *et al.*, (2013). In the present study, the percentage scavenging ability of curcumin was found to be very strong at 45 µg/ml with 91.84% scavenging activity as shown in fig. 5. This is contrary to results reported by Borra *et al.*, (2013), where potential of curcumin to scavenge the ROS species was much less than that of ascorbic acid, concluding that curcumin is not as good a scavenger as ascorbic acid.

Several experimental studies have confirmed curcumin as damaging to DNA in specific cell lines, such as mouse-rat hybrid retina ganglion cells (Lu *et al.*, 2009). In another study conducted by Cao *et al.*, (2006), it was found that curcumin not only proved to be damaging for human mitochondrial DNA, but also for the nuclear genomes in the human hepatoma G2 cells at higher concentration. Consequently, only the minimum concentration of curcumin exhibited DNA protective effects through renaturation of DNA. In the present study, DNA protection assay (fig. 6), is in agreement with above mentioned experimental studies that increasing concentration of curcumin could be damaging to the DNA as a result of which only the minimum concentration produced the protective results.

CONCLUSION

In conclusion, exploitation of *in vitro* techniques has led to the identification of important characteristics of turmeric, assisting in predicting the possible health benefits of turmeric. Confirmation of antibacterial activity illuminates the potential of turmeric to reduce bacterial infection in humans as well as in food. The presence of important phytochemicals showed that turmeric is a valuable spice with many culinary and health benefits while curcumin is a potent antioxidant agent acting as a DNA protectant. Furthermore, the antibacterial effect of curcumin, extraction of essential oils from turmeric and their antifungal effects warrants further exploration and continual experimentation. Curcumin has been validated to have beneficial effect against various chronic inflammatory diseases recently, due to its anti-inflammatory and anti-oxidative effect against a variety of molecular targets as reported by He *et al.*, (2015). Studies on the biological evaluation of curcumin anti-inflammatory and anti-oxidative effect are also recommended.

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