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BIOLOGICAL ACTIVITIES OF INDIAN AYURVEDIC FORMULATION, *PANCHAKOLA*

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Abstract: A large number of studies have proven the efficacy of ayurvedic herbs in the field of health and wellness. The aim of this study was to access the *in-vitro* biological potential of Indian ayurvedic formulation, *Panchakola* which is the equi-proportional mixture of five herbs. The aqueous extract of powdered *panchakola* was characterized for its antioxidant and anti-inflammatory activity. Further, the aqueous extract was analyzed for its antibacterial property against eight bacterial strains including two strains of *Escherichia coli*, one each of *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Aeromonas species* and *Klebsiella oxytoca*. Further, the aqueous extract of *panchakola* was tested against *Candida cells* to reveal its role in antifungal activity. The results revealed that *panchakola* showed 50% DPPH scavenging at 123 µl of extract as compared to the Ascorbic acid's value at 25µl which is known to be a strong antioxidant agent. Also, the highest FRAP value was observed at 500 µl and the lowest was at 50µl i.e. 0.989 mM and 0.419 mM as compared to Ascorbic acid i.e. 1.03 mM and 0.201 mM respectively. The *panchakola* extract showed no anti-inflammatory activity in both the assays upto a maximum amount of 1000 µl. It was found that *panchakola* had strongest inhibitory activity against *E. coli* (76.49%), minimal inhibition against *P. aeruginosa (*28.43%) and no inhibition against *B. subtilis*. Hence, results suggested that it can be used to treat oxidative stressed disorders and also can replace the antibiotics.

Keywords: *Panchakola*, Antioxidant, Antibacterial, Anti-inflammatory, Antifungal, Ayurveda

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INTRODUCTION

Modernization and westernization have made our lifestyle hectic and dull. Western food habits and irregular sleeping patterns has become a major cause of various diseases like hypertension, diabetes, gastrointestinal disorders etc [1]. Allopathic medicines do not have apt response towards a large number of inflammatory, digestion and sexual problems. Therefore, an effective, safe and economical alternative is the need of our [2]. Due to safety and efficacy of medicinal plants, pharmacologists are developing keen interest for research in this area and hence the development of herbal medicines in present scenario [3]. Ayurveda has a strong potential to combat with these emerging problems.

Ayurveda originated in India more than 5000 years ago and is still widely used here. Moreover, its interest has been growing worldwide [4]. To cure the illness, single and polyherbal preparations have been used since ages. In ancient Indian literature, the medicinal properties of several herbal plants have been documented and the preparations are effective in the treatment of several diseases [5, 6].

One of the most popular Ayurvedic formulation, *Panchakola* is used as a general health tonic. Primarily used to cure fever, inflammation, pain, indigestion etc [7], it is a mixture of five components Chavya (root of *Piper chaba*), Pippalimula (root of the *Piper longum*), Chitraka (root of *Plumbago zeylanica*), Pippali (fruit of *Piper longum),* and Sunthi (rhizome of *Zingiber officinale*) in equal proportions as mentioned by Kishor et al. [8].

In this work, we have assessed the *in-vitro* antioxidant, anti-inflammatory, antibacterial and antifungal activities of the aqueous extract of *panchakola.* This is the first time that its aqueous extract is evaluated for biological activities.

MATERIALS AND METHODOLOGY

Chemicals and reagents

The following Ayurvedic formulation obtained from an Ayurvedic doctor (*Hakeem*) from the local Ayurvedic clinic. All solvents and chemicals (analytical grade) used for antioxidant, anti-inflammatory, antibacterial and antifungal assay were purchased from Merck and Himedia. DPPH, BAPNA were purchased from Sigma-Aldrich.

Microorganisms

The bacterial strains investigated are identified strains and were obtained from NCCS, Pune, India. The studied bacterial strains include *Staphylococcus aureus* MTCC 902, *E.coli* MTCC 443, *E.coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Bacillus subtilis* MTCC 736,

Pseudomonas aeruginosa MTCC. All the test bacterial stock cultures were maintained at 4 °C on nutrient agar slants**.**

Preparation of *panchakola* extract

The aqueous extract of *panchakola* was prepared by dissolving 1g of *panchakola* mixture in 20 ml of distilled water (DW). The components of mixture were taken in equal proportion as mentioned previously. The sample was soaked for 2 hours and strained through muslin cloth. The sample was then centrifuged and the supernatant was picked and used as such which served as aqueous extract for the further studies.

Evaluation of Antioxidant Potential of *Panchakola* Extract

DPPH free-radical scavenging activity

The DPPH assay of the *panchakola* aqueous extract was performed as described by the Braca et al. with slight modifications [9]. The reaction mixture contained plant extract at different concentrations and then DPPH solution (0.1 mM in 80% methanol) was added and shaken vigorously. The reaction mixture was then incubated for 30 minutes in dark. Then, the DPPH scavenging activity was measured spectrophotometrically by taking the absorbance at 517 nm against the corresponding test blanks. The percentage scavenging activity of DPPH free radical was calculated using the formula:

Scavenging activity (%) = $[(A_c-A_s)/A_c] \times 100$.

Where A_c and A_s represent the absorbance of control and the absorbance of test samples respectively.

FRAP (Ferric reducing antioxidant power) assay

Ferric reducing antioxidant power (FRAP) assay was performed using the method of Benzie & Strain with minor modifications [10]. A total of 75 µl of sample and 225 µl of distilled water were added to 1.5 ml of freshly prepared FRAP reagent [10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM 2,4,6-tri (2-pyridyl)-*s*-triazine (TPTZ) solution and 1 part of 20 mM $FeCl_3.6H_2O$]. The reaction mixture was incubated in the dark for 4 min. The increase in absorbance with the formation of colored product (ferrous tripyridyltriazine complex) was recorded at 593 nm. FRAP working solution was taken as blank. The antioxidant capacity of the *panchakola* extract was determined based on a calibration curve plotted using $FeSO_4.7H_2O$ at a concentration ranging between 0.125 and 2 mM taking the concentration of $FeSO_4.7H_2O$ mM on x-axis and the absorbance at 593 nm on y-axis. Results were expressed in mM Fe (II)/g of extract. The relative activity of the sample was compared to $_L$-ascorbic acid.

Assessment of Anti-inflammatory activity

Inhibition of albumin denaturation

Albumin denaturation inhibition activity of *panchakola* extract was determined by using the method of Mizushima et al. with slight modifications [11]. The reaction mixture contained *panchakola* extract at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted with small amount of 1N HCl. After that, the samples were incubated at 37°C for 20 min and then heated at 57°C for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percentage inhibition of protein denaturation was calculated by using the given formula:

Percentage inhibition (%) = $(A_c - A_s)/A_c \times 100$

Where A_c is the absorbance of control and A_s is the absorbance of test samples.

Proteinase inhibitory action

The proteinase inhibitory activity of *panchakola* extract was determined by using the method of Erlanger et al. with minor modifications [12]. In this assay, we determined anti-inflammatory potential by measuring the residual enzymatic activity towards the substrate 1.5 mM BAPNA–HCl (N-benzoyl-arginine-p-nitroanilide–HCl) predissolved in 20% Glycerol. The test reaction mixture contained 20 µl trypsin (1 mg/ml in 20 mM Tris–2mM $CaCl_2$, pH 8.2), 100 µl of extract and 80 µl Tris buffer. It was then incubated for 10 min at room temperature. The reaction was initiated by the addition of 500 µl of BAPNA solution. The reaction mixture was further incubated for 10 minutes at room tempertaure. Later, 300 µl of 30% acetic acid solution was added to terminate the reaction. The enzymatic hydrolysis of the BAPNA was determined spectrophotometrically by measuring the release of p-nitroaniline at 410 nm. Activity was defined as activity of test (extract + trypsin) - Activity of negative control (trypsin alone). Activity of trypsin was calculated by using the given formula:

$$\text{Activity (U)} = \frac{\Delta A \times \text{total volume(in ml)} \times D.F \times 10^6}{\varepsilon_{BAPNA} \times \text{Sample volume (in ml)}}$$

Where, ΔA= change in absorbance at 410 nm; D.F.= Dilution Factor; ε_{BAPNA} = Molar extinction coefficient of BAPNA.

Determination of Antibacterial activity

Antibacterial activity of *panchakola* extract was tested against various bacterial strains. Overnight cultures was prepared in Luria broth (LB) media by inoculation with a single colony from agar plates and was then incubated at 37 ºC for 12 h. Overnight cultures were diluted with fresh LB media to approximately 104 colony-forming units (CFU) and incubated at 37ºC for 12-14 hrs in the presence of *panchakola* extract compared to the growth of the control culture in the absence of *panchakola* extract. For each strain, three replicates were used. The mean growth inhibition (MGI) values were obtained spectrophotometrically by taking absorbance at 600 nm. It was converted into the inhibition percentage of growth in relation to the control treatment by using the formula:

$MGI\ (\%) = (A_c - A_s / A_c) \times 100$

Where A_c and A_s represent absorbance of control and treated sample strains respectively.

Determination of Antifungal activity

The minimum inhibitory concentration of Panchakola aqueous extract against *Candida cells (*ATCC 10261 and ATCC 90028) was determined by broth dilution by the method as described by the Clinical and Laboratory Standards Institute (CLSI). Cultures were grown with or without test compounds in the media. Two fold dilutions of the test compound were carried out as an initial step. Also, antifungal activity of aqueous extract in solid media was determined by the Disc Diffusion Method. *Candida* cells (10^5cells/ ml) were inoculated in molten YEPD agar (~ 40ºC) and poured into a petri plates. Filter discs were placed on solid agar and different concentrations of test compounds were applied on the disc in 10µl volume. The average diameter of the zone of inhibition was recorded in millimeters. The experiment was performed in triplicates.

RESULTS

Evaluation of Antioxidant Potential of Panchakola Extract

DPPH free-radical scavenging activity

DPPH is stable and non-physiological radical and most widely used method for screening antioxidant activity of plant extracts [13]. DPPH is reduced to diphenylpicryl hydrazine with plant extracts in a concentration-dependent manner. It is visually noticeable as the colour changes from purple to yellow. *Panchakola* showed DPPH radical scavenging activity in a concentration–dependent manner as shown in the figure 1.

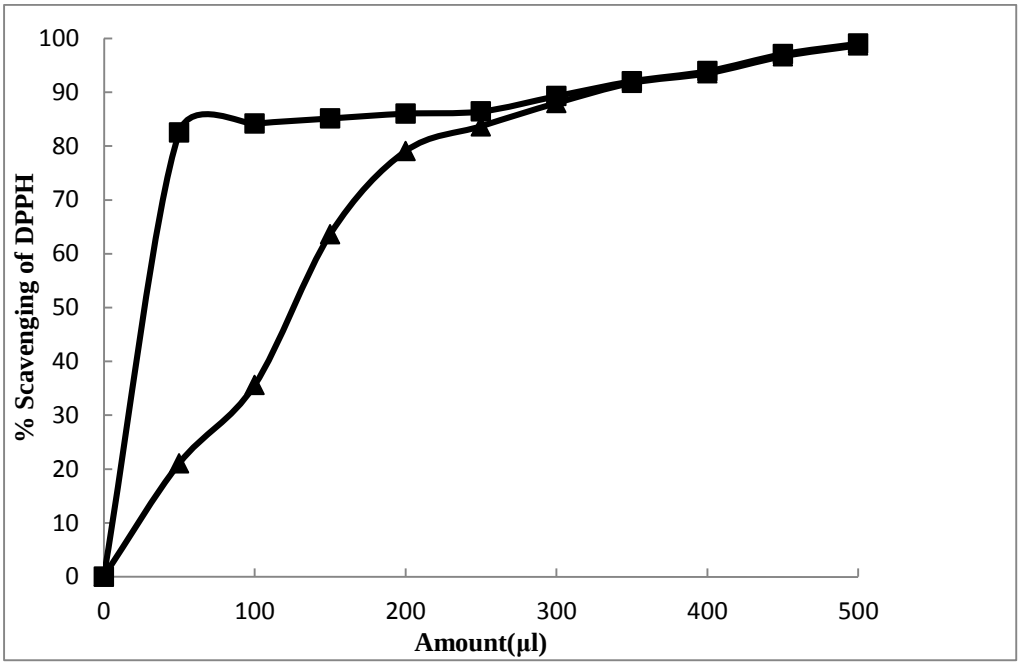

Figure 1: Free radical scavenging activity of *panchakola* aqueous extract was evaluated against DPPH radical by taking varied amount of extract from 100 to 1000 µl and absorbance was recorded at 517 nm against the corresponding test blanks. DPPH radical scavenging activity was plotted between % scavenging activity and amount of panchakola (▲) aqueous extract (µl) taking Ascorbic acid (■) as standard.

FRAP assay

The FRAP assay is a simple, convenient and reproducible method widely employed to determined the total antioxidant activity of biological samples [14]. FRAP is reduced by the compounds that can donate hydrogen atoms to free radicals and convert them into stable non-reactive molecules [15]. As shown in the figure 2, the highest FRAP value was observed at 500 µl and the lowest was at 50µl i.e. 0.989 mM and 0.419 mM as compared to ascorbic acid i.e. 1.03 mM and 0.201 mM respectively. From the observations, it is clear that *panchakola* showed fair antioxidant activity comparable to $_{L}$- ascorbic acid.

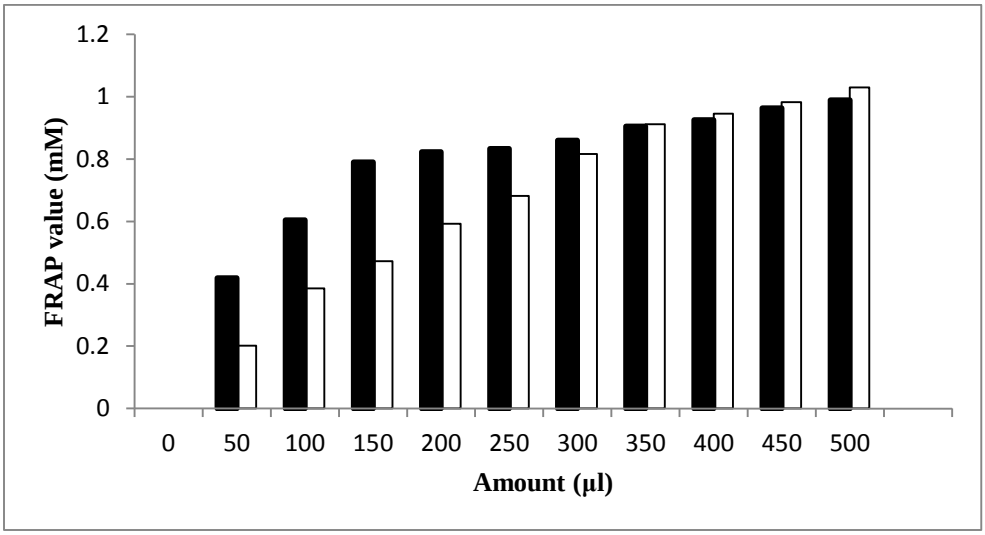

Figure 2: Bar diagrammatic representations of *in vitro* Ferric Reducing Antioxidant Potential (FRAP). The bars represent *panchakola* aqueous extract (■) and a positive control Vitamin C (□). Each bar represents amount of antioxidant required to reduce mM of FRAP reagent.

Assessment of Anti-inflammatory activity

Panchakola was checked for anti-inflammatory activity by proteinase inhibitory and albumin denaturation assay. The results indicate that the *panchakola* extract did not inhibit the proteinase trypsin upto 1000 µl volume. From the results, it was also found that *panchakola* extract was in-effective in inhibiting heat induced albumin denaturation upto 1000 µl volume too.

Determination of Antibacterial activity

Antibacterial assay of the *panchakola* extract was examined against various bacterial strains by accessing the percentage inhibition in presence of *panchakola* compared to the control where only media and cultures were added. The results suggested that *panchakola* exhibits bactericidal property *in-vitro* i.e. the growth of bacteria was inhibited in its presence. It was found that *Panchakola* had strongest inhibitory activity against *E.coli* (MTCC 443) i.e. 76.49% and minimal inhibition against *P. aeruginosa* (MTCC 2453) i.e. 28.43% whereas it showed no inhibition against *B. subtilis* (MTCC736) as shown in figure 3.

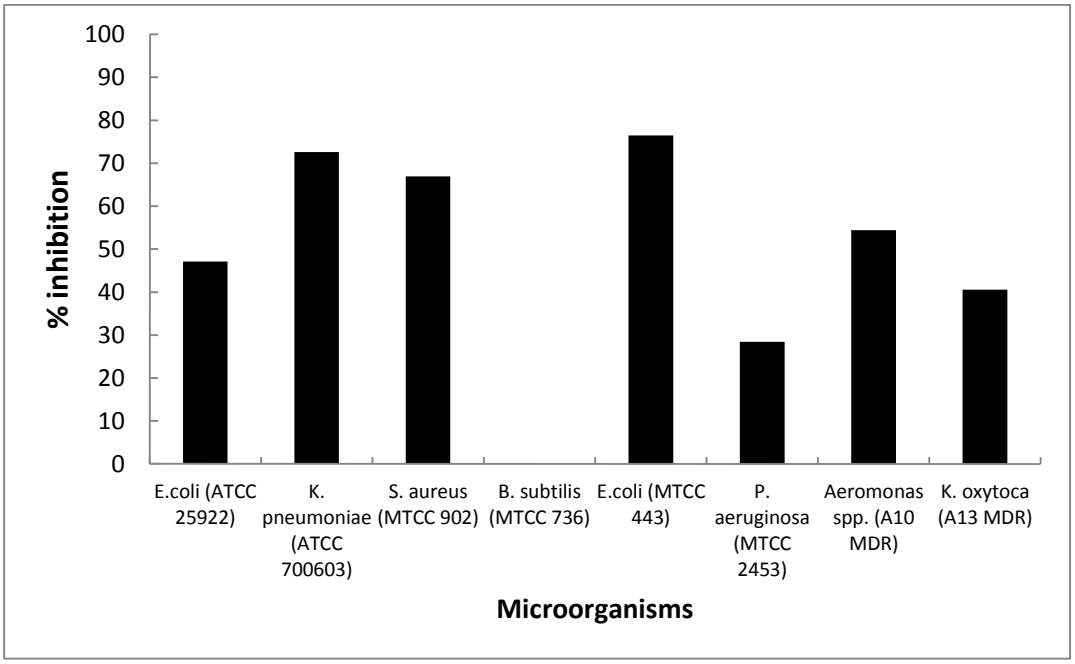

Figure 3: Bar diagrammatic representations of *in vitro* antibacterial activity. The bars represent the percentage mean growth inhibition (■) by aqueous extract of *panchakola* when tested against 8 bacterial strains.

Determination of Antifungal assay

Antifungal assay of the *panchakola* extract was performed against *Candida cells (*ATCC 10261 and ATCC 90028). *Panchakola* extract showed no zone of inhibition upto 1.5mg/ml as well as there is no inhibition in growth of *Candida* cells upto 1mg/ml in broth as well. Our results showed that *panchakola* extract showed no potent anticandidal activity upto 1mg/ml, increased concentration of *panchakola* extract might lead the anticandidal effect.

DISCUSSION

Medicinal plants are being used as an alternate source to get therapeutic compounds based on their medicinal properties. The purpose of this study was to investigate the antioxidant, antibacterial, anti-inflammatory and antifungal activities of Indian Ayurvedic formulation panchakola. The results of the current study have shown that the studied ayurveda is potentially a good source of free-radical scavenging and antibacterial compounds and support the traditional medicinal application.

Many researchers have already been reported that Gram positive bacteria are more susceptible than Gram negative bacteria towards plants extracts [16, 17]. These differences are due to the presence of single layered cell wall in Gram positive bacteria, whereas it is multilayered in the

Gram negative bacteria [18]. Free radicals produced by cells are known for their damaging role and antioxidants protect cells against damage caused by these molecules. The plant extracts are rich in phenolic compounds such as phenolic acids, flavonoids, tannins and phenolic diterpenes [19].

In the proposed study, *panchakola* aqueous extract was evaluated for its antioxidant potential using FRAP reagent and methanolic DPPH reagent. DPPH is unaffected by certain side reactions, such as enzyme inhibition and metal ion chelation because it is very stable free radical [20]. This assay gave information on the reactivity of extract with DPPH which is a stable free radical. DPPH has strong absorption at 517 nm in visible spectroscopy (deep purple color) because of the presence of an odd electron and this color usually gets decolorized when exposed to antioxidant in the solution. If the absorption is lower, the free radical scavenging activity of the extract will be higher [21]. Results suggested that DPPH radical scavenging activity showed dose-dependent inhibition. However, ascorbic acid which was used as positive control showed highest inhibition at the same concentration.

Also the reducing ability of antioxidants against the damages caused by reactive oxygen species can be evaluated using FRAP assay. The antioxidants donate electron and called as reductants and these reductants cause the inactivation of oxidants. Total antioxidant power may be referred analogously to total reducing power [22]. These antioxidant studies proved superior efficacy of *panchakola* extract and is safe and effective intervention for free radical mediated diseases.

The aqueous extract of panchakola exhibited the antibacterial activity against 7 bacterial strains (Figure 3) and the results were expressed in % mean growth inhibition and the obtained values varied from 28.43% to 76.49%.

As *panchakola* is known to have valuable medicinal compounds and contain high antibacterial and antioxidant properties so the ingestion of extract may help to prevent *in vivo* oxidative damage associated with diseases and illnesses and also it can be used to replace the synthetic antibacterial drugs and can further be explored for the isolation of its bioactive compound.

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