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Evaluation of Antioxidant and Antimicrobial Activities of Chitrakadi Vati, a Ayurvedic Formulation

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Abstract

Free radicals are implicated for more than eighty diseases including diabetes mellitus, arthritis, cancer, ageing, etc. in treatment of theses diseases; antioxidant therapy has gained an utmost importance. Current research is now directed towards finding naturally occurring antioxidant of herbal drugs. Antioxidant activity of methanol extract of Chitrakadi Vati was evaluated by using Phosphomolybdenum assay, DPPH radical scavenging assay, superoxide radical scavenging assay and ABTS assay. The total phenolic, total tannins and total flavonoids content were determined. Antibacterial activity was also studied against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Streptococcus pyogenes* by using cup-plate method. Erythromycin was used as standard antibacterial agent. The methanol extract was diluted into different concentration (1, 2, 4, 6, 8, 10 mg/l00 µl) with DMSO. The results of the study revealed that, the Chitrakadi Vati exhibited significant antibacterial activity.

Key words: Chitrakadi vati, Antioxidant, Antibacterial, Erythromycin.

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Introduction

Majority of the diseases are mainly linked to oxidative stress due to free radicals (Gutteridgde, 1995). Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Tiwari, 2001). The most common reactive oxygen species (ROS) include superoxide anion (O^-), hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and reactive hydroxyl (OH^-) radicals. The nitrogen-derived free radicals are nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging physical injury, infection and acquired immunodeficiency syndrome (Joyce, 1987). In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting oxygen scavengers (Buyukokuroglu et al., 2001; Shahidi, 1992). Plant products are being used as source of medicine since long. The medicinal properties of plants have been investigated in recent scientific developments through the world, due to their potent antioxidant activities, no side effects and economic viability (Auudy et al., 2003). Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic, etc. they were

also suggested to be a potential iron chelator (Boyer et al., 1988;Havsteen, 1983; Miller, 1996).

Chitrakadi vati is well known ayurvedic formulation, used traditionally for the as anti-infective agent, as digestive tonic and carminative at 2 to 4 tablets thrice in a day (Anonymous, 2000). Chitrakadi vati consisting of Chitrak (*Plumbago Zeylanicum*), Pippali mula roots (*Piper longum*), Beeja poora (*Citrus medica*), Rock salt, Ajowan seeds, Black pepper roots (Piper nigram), Asafetida (*Ferula foetida*), Trikatu (made up of long pepper, black pepper and ginger; long pepper). The main aim of the study is to investigate the antioxidant and antimicrobial activity of methanol extract Chitrakadi vati.

Materials and Methods

Preparation of tablets

Chitrakadi Vati tablets were prepared by using of chitrak (*Plumbago Zeylanicum*), Pippali mula (*Piper longum*), Beeja poora (*Citrus medica*), Rock salt, Asafetida (*Ferula foetida*), Ajowan seeds, Black pepper roots (Piper nigram), and Trikatu (Combination of long pepper, black pepper and ginger). All the contents were communicated, grind with citrus medica juice and compressed into 500 mg tablets (Anonymous, 2000).

Chemicals

DPPH (1,1- diphenyl-2- picrylhydrazyl), (TBA) thiobarbituric acid, Folin-Ciocalteu reagent, Folin Denis reagent, ABTS (2,2'-azino bis (3-ethylbenzothiazoline-6-sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman -2 carboxylic acid), potassium persulfate and gallic acid were purchased from Sigma Chemical Co. α-Tocopherol (Merck, Mumbai). Ascorbic acid (Biochem Internationals Pvt. Ltd., Bangalore). All other reagents and solvents used were of analytical grade.

Microbial Strains

Bacterial strains were obtained from Microbial Type Culture Collection (MTCC), *Staphylococcus aureus* MTCC 3160, *Escherichia coli* MTCC 40, *Streptococcus pyogenes* MTCC 389 and *Bacillus Subtilis* MTCC 121, procured from Department of Biotechnology, Nagarjuna College of Engineering and Technology, Bangalore.

Preparation of extract for antimicrobial activity

Chitrakadi vati was extracted with methanol by maceration process. The different concentrations (1, 2, 4, 6, 8 and 10 mg/100 µl) were prepared with methanol.

Estimation of total phenolic content

The total phenolic content of the extract was estimated according to the method described by Singleton and Rossi (Singleton VL, 1965). From the stock solution (1 mg/ml) of the drug, suitable quantity was taken into a 25 ml volumetric flask and mixed with 10 ml of water and 1.5 ml of Folin Ciocalteu's reagent. After 5 min, 4 ml of 20% (w/v) sodium carbonate solution was added and volume was made up to 25 ml with double distilled water. The absorbance was recorded at 765 nm, after 30 min. Percentage of total phenolics was calculated from calibration curve of Gallic acid (50-250 µg) plotted by using same procedure and total phenolics were expressed as % Gallic acid.

Estimation total tannins

To the 5 ml of Folin Denis reagent (100g of sodium sulphate + 20g of phosphomalybdic acid +50 ml of phosphoric acid and 750ml of distilled water was refluxed or boiled for 2 hrs and make up the volume 1000 ml with distilled water) mixed 10 ml of 35% sodium carbonate and add different concentrations of methanolic extract of Chitrakadi vati. Then make up the volume to 100 ml in volumetric flask with distilled water. Incubate reacting mixture for 30 min at room temperature and absorbance was recorded at 760 nm. Percentage of total tannins was calculated from calibration curve of tannic acid (100-1000 µg) plotted by using same procedure and total tannins were expressed as % tannic acid (William, 1960).

Estimation of total flavonoids

In a 10 ml volumetric flask add 4 ml of water 1 ml of methanolic extract of Chitrakadi vati keep aside for 5 min. then add 3 ml of 5% sodium nitrite and 0.3 ml of 10% aluminum chloride allow the reaction for 6 min. again add 2 ml of 1M sodium hydroxide. Know make up the volume to 10 ml with distilled water and measure the absorbance of pink chromogen at 510 nm. Percentage of total flavonoids was calculated from calibration curve of Quercetin (100-1000 µg) plotted by using same procedure and total flavonoids were expressed as % quercetin (Chang et al., 2002).

Phosphomolybdenum assay

The assay is based on the reduction of Mo^{VI} to Mo^{V} by the extracts and subsequent formation of a green phosphate/Mo^{V} complex in acidic pH. Separately extracts and formulations were mixed with 3ml of reagent solution (0.6M Sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate), incubated at 95°C for 90 min, cooled to room temperature, and absorbance measured at 695nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid (ASC) using standard plot (Prieto et al., 1999).

DPPH radical scavenging activity

Antiradical activity was measured by a decrease in absorbance at 516 nm in spectrophotometer (Shimadzu model 1601) of methanolic solution of colored DPPH brought about the sample. The plant extract (2-1000µg), ascorbic acid in 4 ml of distilled water was added to a methanolic solution of DPPH (1 mM, 1 ml). The mixture was shaken and allowed to stand at 20 C for 30 min; the absorbance of the resulting solution was measured spectrophotometrically at 517 nm and % inhibition was calculated. Ascorbic acid was used as positive control (Ravishankara et al., 2002; Vani et al., 1997) [15, 16].

ABTS radical scavenging assay

For steady state measurements, 100 μM $ABTS^{\bullet-}$ (prepared by the reaction of 2 mM [$ABTS^{2-}$] was mixed with 0.17 mM potassium persulphate in 20 mM phosphate buffer pH 7.4, kept overnight before use) and mixed with methanolic extract (2-1000 μg/ml) and decrease in absorbance was measured at 734 nm (Re et al., 1999).

Superoxide radical scavenging activity

The assay was based on the capacity of the methanol extract of Chitrakadi vati to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Vani et al., 1997; Bagul et al., 2003). The reaction mixture contained 50 mM phosphate buffer pH 7.6, 20 μg riboflavin, 12 mM EDTA, and NBT 0.1 mg/3 ml was added in the sequence. Reaction mixture was initiated by illuminating the reaction mixture with different concentrations (2-1000 μg) of methanol extract of Chitrakadi vati for 90 sec. immediately after illumination, the absorbance was measured at 590 nm, IC_{50} was calculated. Methanol was used for blank reading. Ascorbic acid was used as positive control.

Antibacterial activity assay

The antibacterial activity was evaluated by employing 24 hrs cultures of *B. subtilis, E. coli, S. aureus and Staphylococcus,* using nutrient agar medium. The bacterial strains were transferred to sterile plates aseptically. The plates were left at room temperature and allowed for solidification. In each plate one well of 6 mm diameter were made using a sterile borer. Accurately 100 μl different dilutions of methanol extract of Chitrakadi vati (1,2, 4, 6, 8, 10 mg) and single concentration of erythromycin (5 mg/ml) solutions were transferred to wells aseptically and labeled accordingly. The plates were incubated at 37 ± 1°C for 24 hrs. The diameter of zone of inhibition surrounding each of wells was recorded (Bradshaw, 1992; Cappuccinos, 1992).

Results and Discussion

Preliminary phytochemical screening of methanol extract of Chitrakadi vati showed the presence of phenolics, flavonoids, tannins, and steroids. Subsequent quantification showed shoed the presence of high amount of total phenolics (2.5μg calculated as gallic acid), and comparable amount of tannins (2.35μg) and total flavonoids (1.78μg) respectively. Phenolic compounds are known to be powerful antioxidant agents. Since Chitrakadi vati contains good amount of total phenolics (2.5μg) it was thought of interest to screen it for its possible antioxidant activity. The antioxidant activity may result from the neutralization of free radical initiating oxidation processes, or from the termination of radical chain reactions. For this reason, the above mentioned methods of antioxidant activity estimation were used. The antioxidant activity may result from the neutralization of free radical initiating oxidation processes, or from the termination of radical chain reactions. Flavonoids are effective scavengers of free radical (Chun et al., 2003). Polyphenols are well documented to have microbicide bacteria (Scalbert, 1991; Cowan, 1999). Oxidized polyphenols also have inhibitory activity against bacterial growth (Cowan, 1999; Field, 1992).

Free radical scavenging of phenolic compounds is an important property underlying their various biological and pharmacological activities. The Phosphomolybdenum assay is based on the reduction of Mo^{VI} to Mo^{V} by antioxidant compounds and a formation of green phosphate/ Mo^{V} complex with a maximal absorption at 695 nm and it was efficient to extend its application to plants polyphenols (Mollar et al., 1999). Total antioxidant capacity of Chitrakadi vati was found to be 3.25 μg. The methanolic extract was able to reduce the stable radical DPPH to the colored diphenylpicrylhydrazine. Both extracts and formulation exhibited a concentration-dependent DPPH radical scavenging activity but the formulation showed more potent activity when compared to extracts (Graph 1). Proton radical-scavenging action is one mechanism for oxidation. DPPH has a proton free radical and shows characteristic absorption at 517 nm (purple). When it encounters proton radical scavenger, its purple color fades rapidly (Yamagushi et al., 1998; Soares et al., 1997), suggesting that antioxidant activity of methanol extract of Chitrakadi vati is due to its proton donating ability.

Methanolic extract of Chitrakadi vati was found to have comparable activity to standard ASC in scavenging 100μM ABTS (Graph 2). For kinetic studies, the concentration of ABTS was kept at 100 μM. In the absence of the formulation, the ABTS signal did not show any decay and remained stable (Re et al., 1999). However, in the presence of the methanolic extract of Chitrakadi Vati the absorption due to the ABTS decayed completed in 20 seconds. This absorption time plot was fitted to a single exponential function to get observed decay rate constant, which was found to increase with increasing concentration of extract. The superoxide radical activity of the Ayurvedic formulation increased with the increase in concentration (Graph 3). Superoxide radicals are generated during the normal physiological process mainly in mitochondria. Although superoxide anion is by itself as weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to the oxidative stress (Bagul et al., 2003; Beauchamp, 1999) the capacity of different concentration of extract to scavenge superoxide radical reveals that the extract of formulation possess superoxide dismutase like activity. Although the activity was found to be lower than scavenging activity of ascorbic acid in entire dosage ranges.

Antibacterial assay

Results of antibacterial activity of different dilutions methanol extract Chitrakadi vati were measured in terms of zone of inhibition (Table 1). It revealed that significant antibacterial activity was showed against bacterial strains like *Escherichia coli, staphylococcus aureus, Bacillus subtilis,* and *Streptococcus pyogenes* in comparison with

standards erythromycin. Chitrak promotes digestive power and appetite, uses in fever, oedema, leprosy, scabies etc (Nadkarni, 1999). Trikatu made up of long pepper, black pepper and ginger; long pepper is laxative, antiasthamatic, anti-infective agent in urinary tract infection, carminative, aphrodisiac and analgesic (Yoganarasimhan, 2000). Black pepper traditionally used for constipation, piles, colic, as diuretic and analgesic (Nadkarni, 1999). Ginger is used in cardiac diseases and as carminative (Nadkarni,1999). This study justifies the traditional claim of Chitrakadi vati as anti-infective agent.

This study justifies the traditional claim of Chitrakadi vati as anti-infective agent. Antibacterial activity may be due the presence of polyphenols present in the formulation (Scalbert, 1991). Polyphenols have been reported to exhibit antibacterial activities (Haslam, 1996). The inhibition of microorganisms by phenolic compounds may be due to iron deprivation or hydrogen bounding with vital protein such as microbial enzymes (Scalbert, 1991). Oxidized condensation of phenols may result in the toxification of microorganisms. These supports the fact polyphenols may be responsible for the antibacterial activity of Chitrakadi vati.

Conclusions

Results suggest that the methanol extract from Chitrakadi vati presents antimicrobial activity against Gram-positive bacteria and Gram-negative bacteria, and may be due to the presence of phenols, saponins, flavonoids in its chemical constitution. This study presents the first description of the antimicrobial activity of the crude methanol extract of Chitrakadi vati.

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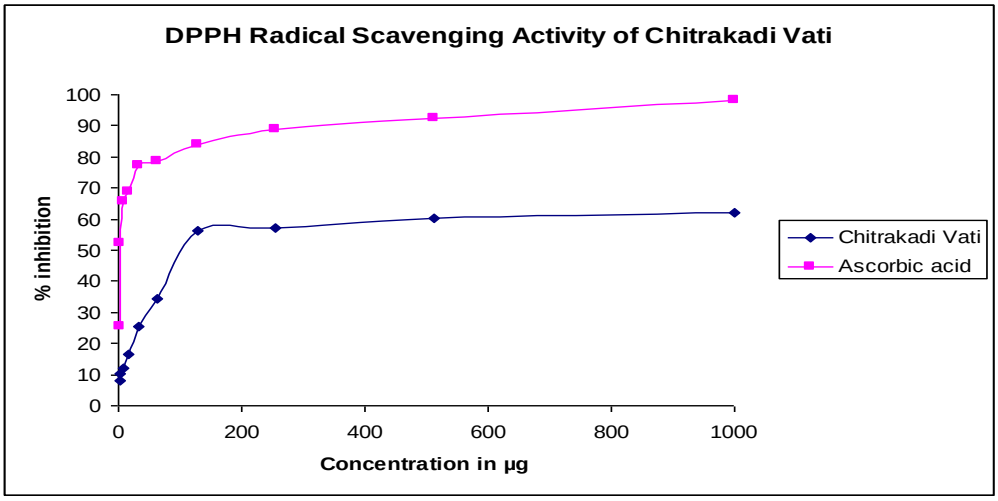
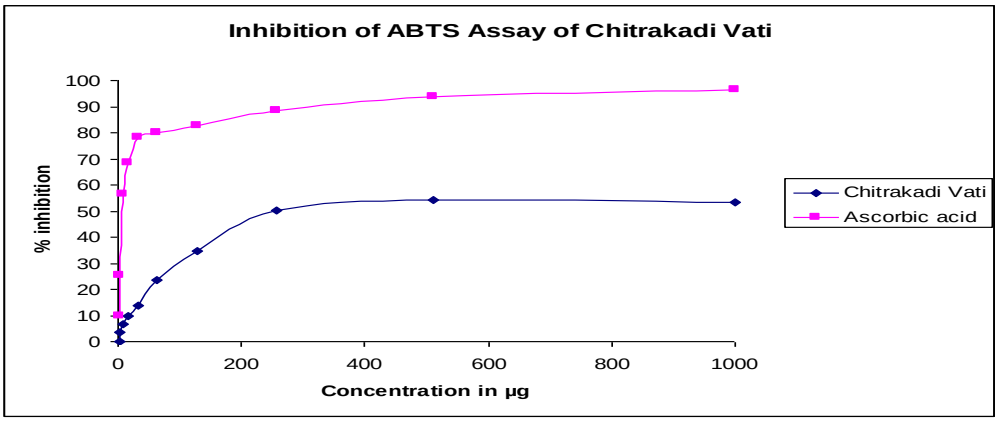
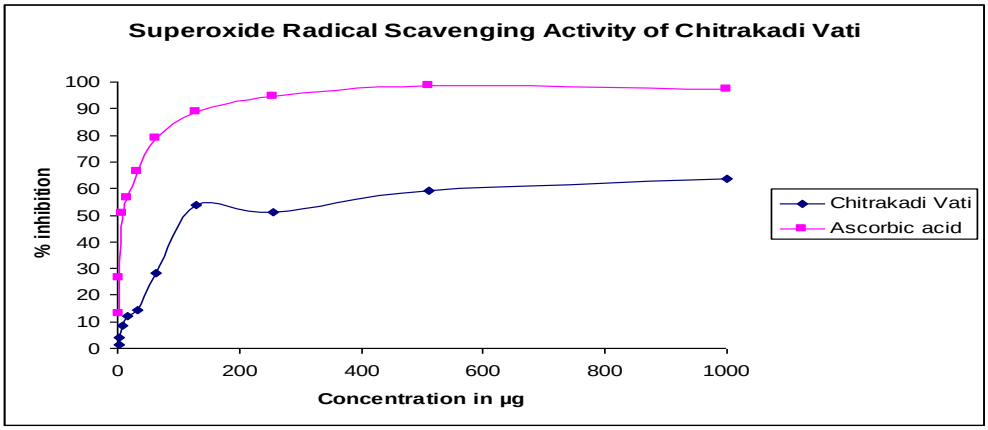


Graph 1



Graph 2



Graph 3

Table 1. Antibacterial activity of methanol extract of Chitrakadi vati

Microorganisms	**Zone of Inhibition of methanol extract in mm**						
	1 mg	**2 mg**	**4 mg**	**6 mg**	**8 mg**	**10 mg**	**Erythromycin 5 µg/100 µl**
E. coli	5	14	10	8	17	24	18
S. aureus	4	6	10	12	14	16	19
B. subtilis	4	6	4	8	10	15	20
S. pyogenes	5	8	8	10	12	16	17

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