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In vitro Antibacterial Activity of *Camellia sinensis* and *Andrographis paniculata* against Vancomycin Resistant *Enterococci*

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ABSTRACT

The herbal extracts of *Camellia sinensis* (*Cs*) and *Andrographis paniculata* (*Ap*) with standard F/P ratio (flavonoid/ polyphenol) of 0.26-0.27 were used for evaluation of anti-microbial efficacy against Vancomycin Resistant *Enterococci* (VRE). The bioactivity fingerprints useful for standardizing both extracts were found to be coherent with respect to their Diphenyl picryl hydrazyl radical scavenging potential (IC_{50} = ~ 30µg/ml). *In vitro* efficacy analysis of *Cs* and *Ap* extracts using exponentially growing culture of VRE revealed Minimum Inhibitory Concentration (MIC) as 200µg/mL and 50µg/mL respectively. The comparative analysis of their Minimum Bactericidal Concentration (MBC) revealed that 5X concentration of *Cs* as against 10X concentration of *Ap* was required to achieve zero microbial count. Such analysis indicated that both extracts would be useful for future analysis. *In vivo* pre-clinical evaluation studies are underway for utilization of identified herbal leads for trials on humans.

Keywords: *Camellia sinensis, Andrographis paniculata,* Vancomycin, *Enterococci,* Minimum Inhibitory Concentrations, Synergistic, Bactericidal.

INTRODUCTION

The irrational use of antibiotics has resulted in evolution of multi-drug resistant (MDR) bacteria thereby widening the spectra of untreatable nosocomial bacterial infections like *Staphylococcus aureus*, *Enterococcus spp., Acinetobacter baumannii* and *Escherichia coli* etc. The average growth rate of increase of such infections was reported to be 4% globally. [1-2] Many infections including blood stream, surgical wounds, urinary tract and respiratory infections etc. fall under this category. [3- 4] *Enterococci* are natural inhabitants of the oral cavity, gut and female genital tract in primates. One of the well-known Multi-Drug Resistant (MDR) genuses of *Enterococci* is Vancomycin Resistant *Enterococci* (VRE) that confers antibiotic resistance due to over-expression of *van A* and *van B* genes. Such MDR profiles have been extensively found in VRE species like *Enterococcus faecalis* and *Enterococcus faecium*. The evolution trends of these species also indicate their probable Pan-Drug Resistance (PDR) capability. [5-6] The present day pharmaceutical industry is not able to face the challenge posed by such newly emerging antibiotic resistant pathogenic and opportunistic microorganisms. The current analysis of Nosocomial

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Infections Surveillance Report revealed that 28 % of all nosocomial infections are attributed to the Vancomycin Resistant Enterococcal strains itself. [7-8] With present emergence rate, there is an urgent need of novel holistic interventions to manage such infections.

An analysis conducted by World Health Organization (WHO) revealed that more than 80% of the global population still relies primarily on herbal medicines as the direct/ indirect primary source of their health care needs. [9] Nearly 6000 Indigenous plants were reported to be useful in traditional, folk and herbal medicine. [10] *Juniperus communis, Lavandula angustifolia,* and *Pelargonium graveolens* have been reported to provide herbal alternatives for conferring resistance in VRE. [7] XRD, SEM, EDAX and FTIR are important techniques often used for standardization of herbals. [11] However, due to lack of bioprospection data for herbals aimed towards multiple physiological targets affected by micro-organisms, new herbal informatics approaches are needed. [12] The previous *in silico* bioprospection study conducted by our research group targeting Vancomycin Resistant *Enterococci* (VRE) revealed *Camellia sinensis* and *Andrographis paniculata* as two potential leads from a database of 50 plants. The present study aims to carry out chemical and bioactivity based characterization/ standardization of these nutraceuticals and analysis of their efficacy at *in vitro* level. [13] Such study will provide an insight into the probable clinical utility of these nutraceuticals with respect to human trials in future, if any.

MATERIALS AND METHODS

Bacterial Strain

The biological samples (urine/stool/blood) were collected from various regional laboratories of National Centre for Disease Control [NCDC], New Delhi. The clinical strains of VRE were isolated using repeated sub culturing on selective media *i.e.*, Blood Agar. The isolated bacterial species were further subcultured on Blood agar and Mueller Hinton agar, at 37°C for 24 h. The isolated and purified cultures were identified through their specific characteristics, that is, morphological and biochemical, as per standard protocols. [14]

Collection and Authentication of Herbals

Camellia sinensis (Cs) and *Andrographis paniculata (Ap)* were collected from the cultivated field areas of Uttarakhand at a latitude and longitude: 29.1500° N & 75.7000° E to 30.3300°N & 78.0600°E, having localized tropical weather conditions promoting cultivation. The selected herbal samples were collected in December, 2013 (Temperature ~ 10°C). Collected specimens were identified and authenticated by a known botanist, Dr. Rajesh Arora, DRDO with a specimen deposited in the repository of INMAS [voucher specimen No. INM-11611-A & INM-31911-A].

Extraction and Quality Control of Herbals

The shade dried leaves (500 g) were mechanically powdered & filtered using sieve size 125, followed by evaluation of plant material based quality measures. [15] The filtered powdered material (consistency: very fine, sieve size-125) was exhaustively extracted using 1:1 aqueous: ethanol [1L, b.p. between 70-80°C] continuously for 4 - 5 hours (temperature ~ 55°C) with continuous condensation & collection of marc. The filtered marc was refluxed (below 60°C) in Soxhlet extractor (Buchi Labortechnik, India, 2008) with aquo-alcoholic solvent (1:1) till viscous extracts formed without any charring. The residual solvent of concentrated extracts was evaporated under vacuum in rotary evaporator (Cole Parmer, India, 2001). The crude extract obtained was lyophilized (80 to 200 mTorr; Temp: -30 to -80°C) in Lyophilizer (FTS System, USA, 2009). The freeze extracts were spray dried to develop amorphous powder (muddy brown in color), aliquots were stored individually at 4°C in cryovials, designated as PTRC-31911-A (*Cs*) & PTRC-11611-A (*Ap*) respectively (solubility tested in ethanol, water, 1:1 aquo : alcohol, peptone water). Percentage yield and moisture content of the extract was determined using standard protocols. [16]

Phytochemical Fingerprinting

Qualitative Phytochemical Analysis

The phytochemical fingerprint profiles (qualitative) of PTRC-31911-A & PTRC-11611-A were developed using alkaloid estimation [Wagner's methodology]; tannins [$FeCl_3$ based testing], saponins [Froth Test], flavonoids [Alkaline Reagent Test], terpenoids [colorimetric terpenoids assay], glycosides [Acetic Acid Test], anthraquinones [colorimetric anthraquinone assay], fats [Spot Test], proteins [Biuret Assay] and reducing sugars [Benedict's Assay]. [17-20]

Quantitative Phytochemical Analysis

Polyphenolic Content Estimation

1 ml of each of PTRC-31911-A & PTRC-11611-A extracts was mixed with 0.1 ml of 1N Folin Ciocalteau reagent. The solution was allowed to stand at room temperature for 15 minutes. Then, 5 ml of 20% sodium carbonate solution was added. The solution was allowed to stand for another 30 minutes at room temperature. Finally, the absorbance was recorded at 760 nm. The same set of protocol was used for Gallic acid, which was used as the standard required for plotting of the calibration curve. [21]

Flavonoid Content Estimation

1 ml of each of PTRC-31911-A & PTRC-11611-A extracts was mixed with 1 ml of methanol. The solution was then mixed with 0.5 ml of Aluminium Chloride (1.2%) and 0.5 ml of Potassium acetate (120 mM). The solution was allowed to stand for 30 minutes at room temperature. Finally, the absorbance was recorded at 415 nm. The same set of protocol was used for Quercetin, which was used as the standard required for plotting of the calibration curve. [22]

Bioactivity fingerprint based functional analysis

The bioactivity based functional profile of the PTRC-31911-A and PTRC-11611-A was developed using IC_X (~10-90) analysis in DPPH (1, 1-diphenyl-2-picryl

hydrazyl) scavenging and Nitric oxide ion scavenging. In the former assay, various concentrations of said extracts and ascorbic acid~ standard (2 to 64µg/ml) [50µL sample made up to 3 ml using MeOH] were developed, 150µl of DPPH solution [4.3 mg in 3.3 ml MeOH, absorbance (λ = 517 nm) of stock solution diluted up to 3 ml using MeOH was treated as control reference] was added and O.D. was taken after 15 minutes at 517 nm, using methanol as blank. In the latter assay, various concentrations of both extracts & standard [50µL sample made up to 150µL using MeOH] were mixed with 2 ml of sodium nitroprusside (10mM in phosphate buffer saline; generates nitric oxide at physiological pH which interacts with oxygen to generate nitrite ions). [23-24] The reaction mixture was incubated at 25°C for 150 min followed by addition of 5ml of Griess reagent and OD was recorded at 546nm. O.D. in both cases was recorded using UV-Vis spectrophotometer [Perkin Elmer, USA, 2001].

***In vitro* Antibacterial Activity against VRE**

Minimum Inhibitory Concentration (MIC) for each of the selected plant extracts was performed using standard method. [25] Stock solution of the plant extracts was prepared with a concentration (10mg/ml). 100µl of overnight grown stock culture of VRE (~10^8 C.F.U/ml) was inoculated into various concentrations of the test extracts ranging from 6.25 to 1000µg/ml. All the inoculated test tubes were incubated at 37°C for 24 hours. The MIC was established by determining the lowest extract concentration corresponding to turbidity in the test tubes (as matched with Mac Farland' solution). The confirmatory analysis was done by culturing on Blood agar plates at 37°C for 24 hrs and assessing the colony count.On the basis of the probable colonies in the inoculum, killing index was determined for every concentration of the dilutions taken using the following formula.

$$\textit{Killing index} = \frac{Probable colonies - Surviving colonies}{Probable colonies}$$

Where number of colonies killed is given by the difference of probable colonies and the number of colonies obtained at that concentration. Absorbance of 100µl of VRE inoculum (~ 10^8 CFU/ml) was found to be ~ 0.8 *i.e.*, actively growing.

RESULTS

Extraction- Quality Control parameters

The ash value of the whole plant of *Camellia sinensis* and *Andrographis paniculata* was 3.5% and 5.7% respectively. Moisture content in the whole plant of *Camellia sinensis* and *Andrographis paniculata* was 8.4% and 7.2% respectively. It was found that the moisture content of the plant extracts was 10 folds lower than the whole plant moisture content values.*Camellia sinensis* extract was found to have a greater percentage of extractive value (91%) followed by 56% as observed in *Andrographis paniculata* (Table 1).

Qualitative Phytochemical Analysis

It is evident from Table 2 that *Camellia sinensis* and *Andrographis paniculata* serve as a good source of diverse phytoconstituents.

Quantitative Phytochemical analysis of Plant extracts

Camellia sinensis and *Andrographis paniculata* extracts were also found to contain a considerable amount of Polyphenol and flavonoids, with polyphenol being in the proportion of 39.934mg/g and 28.4 mg/g respectively and flavonoids being 10.65 mg/g and 7.8 mg/g respectively. The standardized value of Flavonoid: Polyphenol F/P ratio lies between 0.26 - 0.27, an indicator to control batch-to-batch variation (Table 2).

Table 1: Quality Control Analysis

Quality Control Parameter	PTRC-11611-A	PTRC-31911-A
Sieve Size	Very Fine consistency, Sieve Size 125	Very Fine consistency, Sieve Size 125
Foreign Matter	0.034%	0.028%
Macroscopic Examination		
a. Size	Length : 4 mm, Width : 1.5 mm	Length : 5 mm, Width : 2.3 mm
b. Colour	Pale Green	Green
c. Surface Texture	Hard	Soft and Smooth
d. Odour	Distinct	Distinct, Fruity
e. Taste	Tasteless	Sweet
Total Ash Content	5.7%	3.5%
Moisture Content	7.2%	8.4%
% Yield	91%	56%

Table 2: Qualitative and Quantitative Estimation of PTRC-31911-A (Cs) & PTRC-11611-A (Ap)

Qualitative Estimation	Cs	Ap
Alkaloids	+	+++
Flavanoids	+	++
Terpenoids	+	+
Phenolics	-	-
Carbohydrates (Reducing Sugar)	+	++
Proteins	-	+
Fats	-	-
Saponins	+	+
Anthraquinones	+++	++
Glycosides	+++	++
Quantitative Estimation		
Polyphenolic content (w/w of dried extract)	28.4 mg/g	39.934 mg/g
Flavanoid content (w/w of dried extract)	7.8 mg/g	10.65 mg/g

Bioactivity Fingerprinting of Plant Extracts

Bioactivity fingerprinting of the extracts revealed that *Camellia sinensis* and *Andrographis paniculata* extracts have considerable free radical scavenging activity also, with IC_{50} for DPPH Scavenging is 30.332µg/ml and 30.35µg/ml respectively; IC_{25} for Nitric Oxide Scavenging Potential is 112.723µg/ml for *Camellia sinensis* extract, whereas *Andrographis paniculata* extract did not show any significant Nitric Oxide scavenging activity (Figure 1).

***In vitro* Antibacterial Activity against VRE**

The results showed that both the extracts are potent inhibitors of VRE with respective MIC values of *Camellia sinensis and Andrographis paniculata* as 200µg/ml and 50µg/ml respectively. The respective

Minimum Bactericidal Concentration (MBC) was 1000µg/ml and 500µg/ml. Minimum Inhibitory Concentration of both the extracts is depicted in Figure 2.

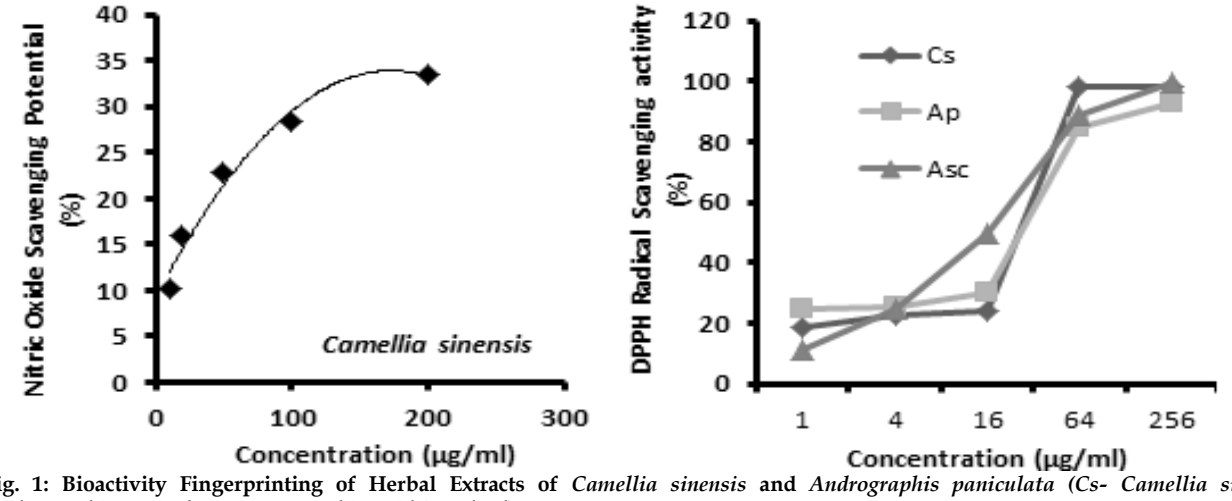

Fig. 1: Bioactivity Fingerprinting of Herbal Extracts of *Camellia sinensis* and *Andrographis paniculata* (Cs- *Camellia sinensis*; Ap- *Andrographis paniculata*; Asc- Ascorbic Acid Standard)

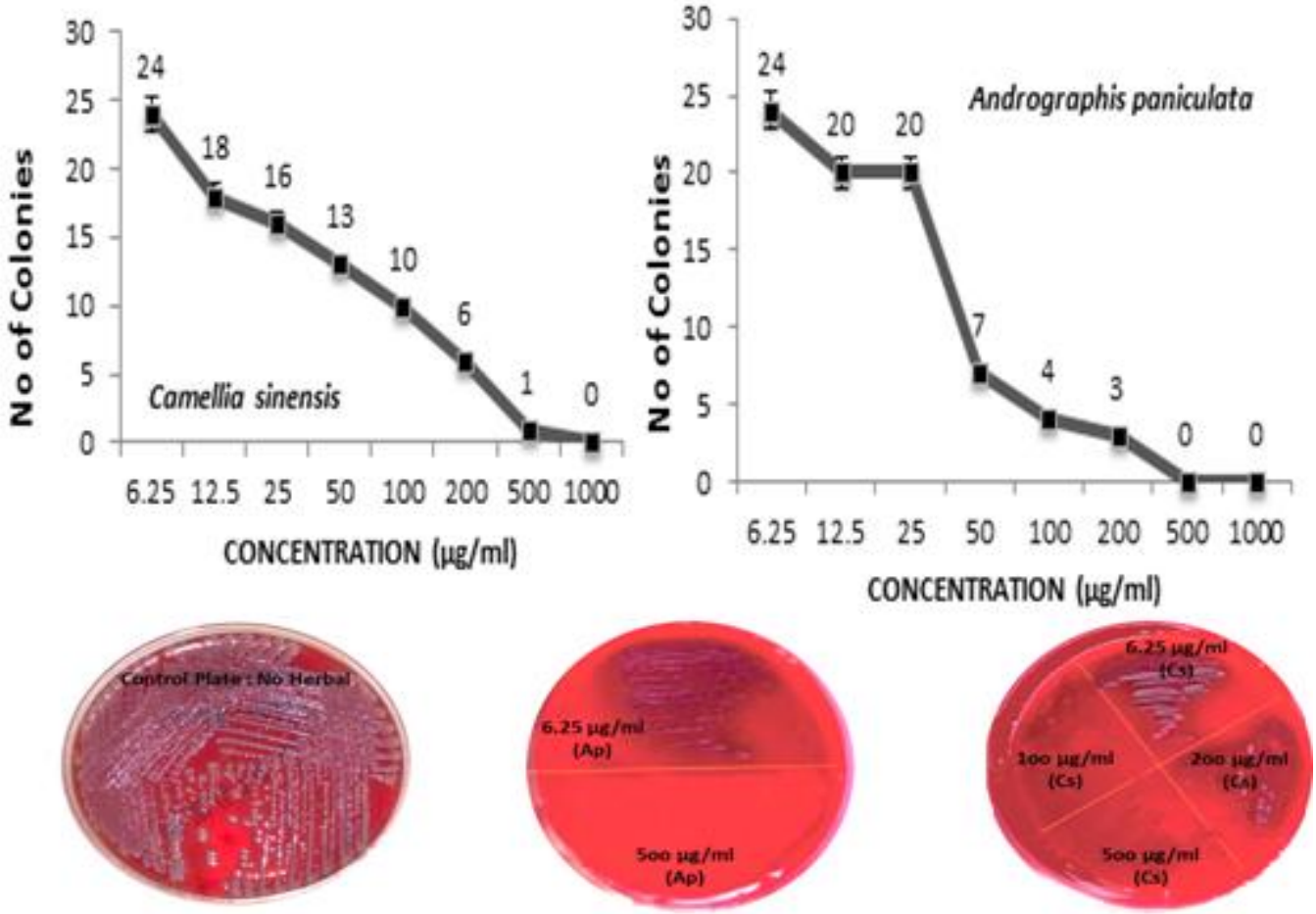

Fig. 2: Minimum Inhibitory Concentration of *Camellia sinensis* (Cs) and *Andrographis paniculata* (Ap) extract at different concentrations, ranging from 6.25 to 1000 µg/ml

DISCUSSION

Control of infections acquired in hospitals and communities caused by multi-drug resistant bacteria pose a major public health concern globally. In the past few decades, superbugs like Methicillin Resistant *Staphylococcus aureus* (MRSA), NDM-1 *Escherichia coli*, VRE have emerged as devastating pathogens in both hospitals and community settings. [26] These bacteria are often implicated in a variety of nosocomial infections including bacteremia, urinary tract infections, and nosocomial pneumonia. Treatment of these infections is often very difficult due to cross-resistance of these bacteria with a large group of antibiotics, so it seems reasonable to explore new sources of natural compounds with bactericidal potential.

The present study has explored *in vitro* potential of nutraceuticals pre-identified using *in silico* herbal bioprospection model [27] i.e., *Camellia sinensis* (Cs) and

Andrographis paniculata (Ap). *C. sinensis* has been proved to possess medicinal and health promotion properties, including the ability to inhibit the growth of some types of pathogenic bacteria. [27] Both extracts were prepared using aquo-ethanolic solvent system with intermediate polarity. The flavonoid/ polyphenol ratio in both extracts was found to be in the range of 0.26-0.27 indicating their similar functional potential. Bioactivity fingerprint based standardization revealed that DPPH free radical scavenging potential can be utilized as targeted assay to minimize batch-to-batch variation and indicator for their shelf life. Both extracts exhibited similar IC_{50} = ~ 30µg/mL.

In this study we found that the MIC of *Camellia sinensis* and *Andrographis paniculata* extract was found to be 200µg/mL against exponentially growing VRE. The anti-bacterial activity of both the extracts was found to be comparable with respect to reported values for standard antibiotic *i.e*. Gentamycin and Ampicillin with respective MICs as 103µg/mL and 160µg/mL. [28-29] These finding are consistent with other studies that have previously been reported anti-bacterial activity of *Camellia sinensis* (Green Tea) and *Andrographis paniculata* against resistant bacteria strains such as MRSA, and MDR-*Pseudomonas aeruginosa.* [27-29] Further such antimicrobial potential of green tea was attributed to the presence of various polyphenolic components including epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate. [26-27] It was also reported that the main component of tea polyphenols, epigallocatechingallate can reverse methicillin resistance of MRSA by inhibiting the synthesis of Penicillin Binding Protein (PBP). Epigallocatechin gallate not only increases the activity of β-lactams but also increases the activity of non-β-lactam cell wall biosynthesis inhibitors. Mechanism of action of green tea leaves extract has been proposed that green tea can prevent the attachment of pathogenic bacteria on the host cell membrane. Thus, green tea extract inhibits the adhesion of bacteria on host cell surface membranes and acts as a potential anti-adhesive agent. [30]

Epigallocatechingallate, which is a type of proanthocyanidin from green tea has also been reported to interact with the outer membrane bacterial and may prevent the adhesion to mammalian epithelial cells (HEp-2), and probably without alteration in mammalian epithelial cells. [27, 30] Another possible mechanism is green tea extract may affect the activity of dihydrofolatereductase, an enzyme that is needed by pathogenic bacteria to synthesize purine and pyrimidine as well as increase the thickness of the epidermis. [28] Such studies indicated that Cs could be used as one of the important medicinal value herbals against VRE too.

The antibacterial activity (MIC against VRE) of *A. paniculata* extract was found to be 50µg/mL which was nearly five times more effective than *C. sinensis* at *in vitro* level. Whilst many studies have isolated and characterized *A. paniculata* compounds, no study has ever determined the antimicrobial activity of isolated compounds so far. 3-O-β-D-glycosyl-14-deoxyandrographolide and 14 deoxyandrographolide were isolated as active principles, which may serve as lead for the development of new pharmaceuticals that might address the unmet therapeutic needs to cure chronic bacterial infections effectively. [29]

The comparative analysis of both extracts in terms of their bactericidal potential against VRE revealed that *C. sinensis* achieves such potential at 5X concentration as compared to its MIC while in case of *A. paniculata*, the factor is 10X. This finding concludes that at lower concentrations, higher bacteriostatic potential was observed in case of *A. paniculata* while higher bactericidal potential was observed in case of *C. sinensis* at higher concentrations. Thus, both herbal candidates qualify for future *in vivo* pre-clinical evaluation and toxicity analysis. In addition, further investigation is needed to determine the bioavailability of the active compounds and to determine the dose and toxicity before it can be used as therapeutic agents. The results also open the door for novel mechanisms of synergistic actions where different combinations of potent drugs can be made highly efficacious involving the synergistic interactions among the drugs.

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