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INHIBITORY EFFECT OF SOME INDIGENOUS MEDICINAL PLANTS AGAINST HUMAN PATHOGENIC BACTERIA *SALMONELLA* AND FUNGI *CANDIDA ALBICANS:* A PHENOMENOUS APPROACH

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ABSTRACT

Plant based antimicrobial compounds have enormous therapeutically potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobial. *Salmonella serovars* is the causative agent of typhoid fever a contagious infection of the intestines that affects the whole body. Whereas *Candida albicans* is a diploid fungus (a form of yeast) and a causal agent of opportunistic oral and genital infections in humans. Based on the result obtained in this study, the herbal plant extracts could be much more relevant for use as antimicrobial agent. Synergistic effect experienced in combination of some plant sources for use as antimicrobial agent. The Trifala showed 10.6 mm zone of inhibition against *Salmonella* serovars as compared to Harro (9.6mm) whereas in case of *Candida Albicans*, Trifla showed (13.7mm) more zone of inhibition as compared to Harro (9.2mm). Thus, the use of this combination of plant source is recommended for use, combined usage practiced among the indigenes. Apart from combination of plant extracts like Trifala, some individual plants like Kalonji have also shown significant antimicrobial activity against *Salmonella*. Future research on this study can however concentrate on optimizing clinically the chemotherapeutic processes, treatment and control of these common food-borne enteric infections like typhoid in Madhya Pradesh and

other parts of the country where the plants can be easily cultivated or otherwise commercialized for pharmaceutical purpose.

Key Words: Antibiotics, Typhoid, *Salmonella typhimurium*, *Candida albicans.*

INTRODUCTION

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. Since the discovery of these antibiotics and their uses as chemotherapeutic agents there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms (Harbottle *et al*., 2006). The worldwide emergence of *Escherichia coli, Klebsiella pneumoniae, Haemophilus* and many other ß-lactamase producers has become a major therapeutic problem. Multi-drug resistant strains of *E. coli* and *K. pneumoniae* are widely distributed in hospitals and are increasingly being isolated from community acquired infections (Khan & Musharrof, 2004; Akram *et al*., 2007). *Candida albicans*, also a nosocomial pathogen, has been reported to account for 50-70% cases of invasive candidiasis (Paula *et al*., 2006). Alarmingly, the incidence of nosocomial candidemia has risen sharply in the last decade (Kao *et al*., 1999). All this has resulted in severe consequences including increased cost of medicines and mortality of patients. Thus, in light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. However, the past record of rapid, widespread emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy (Coates *et al*., 2002). For this reason, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs against MDR microbe strains (Braga *et al*., 2005). For thousands of years, natural products have been used in traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs. The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief. It is estimated that local communities have used about 10% of all flowering plants on earth to treat various infections, although only 1% have gained recognition by modern scientists (Kafaru, 1994). Owing to their popular use as remedies for many infectious diseases, searches for plants containing antimicrobial substances are frequent (Betoni *et al.*, 2006). Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties (Lewis and Ausubel, 2006). A number of phytotherapy

manuals have mentioned various medicinal plants for treating infectious diseases due to their availability, fewer side effects and reduced toxicity (Lee *et al*., 2007). There are several reports on the antimicrobial activity of different herbal extracts (Bonjar, 2004; De Boer, 2005). Many plants have been found to cure urinary tract infections, gastrointestinal disorders, respiratory diseases and cutaneous infections (Brantner, 1994; Somchit *et al*., 2003). Cytotoxic compounds have been isolated from the species of *Vismia* (Hussein *et al*., 2003). Antibacterial activity of the essential oil as well as eugenol purified from *Ocimum gratissimum* to treat pneumonia, diarrhea and conjunctivitis has also been reported earlier (Nakamura *et al*., 1999). According to the WHO, medicinal plants would be the best source for obtaining variety of drugs (Santos *et al*., 1995). These evidences contribute to support and quantify the importance of screening natural products. The aim of the present study was to investigate the antibacterial and antifungal activity of ethanolic extracts of *Azadirachta indica, Emblica officinalis, Nigella sativa, Terminalia chebula, Triphala* against multi-drug resistant strains isolated from nosocomial and community acquired infections.

MATERIAL AND METHODS

The present study was carried out at the Environmental Biotechnology Laboratory, Department of Post-Graduate Studies and Research in Biological Science, R.D University, Jabalpur during the period from February to June, 2011. The methodology followed is as under-

Collection of Medicinal Plants

Plant materials were collected from different sites of Jabalpur region, Madhya Pradesh. The plants were identified and confirmed with authentic. The plant selected for the present study was *Azadirachta indica* (leaves), *Emblica officinalis* (fruits), *Terminelia chebula* (fruits), *Terminalia Belerica* (fruits) (collected for the preparation of Triphala) and *Nigela sativa* (seeds). Fresh leaves of Neem were collected from forests and rest of the fruits were collected from local market and washed individually under running tap water to remove soil and dirt particles and shade dried under room temperature. The dried plant parts were grained into coarse powdered with the help of mortar and pestle and used for further investigation.

Preparation of Plants Extracts

***Azadirachta Indica* (NEEM)**

Ethanolic extract of Neem was prepared by dissolving 20, 30 and 50 grams of dried leave powder in 100ml of ethanol separately and left for 72 hrs at room temperature. After that it

was filtrated through a sterile filter paper (Whatman-1), each of these solvent extract were preserved at 4^0C in screw cap bottles for further study.

***Nigela Sativa* (KALONJI)**

Black cumin seeds were bought from the local market and sorted for separation of dirt and unwanted material. The seeds were dried at 40^0C overnight and were ground to powder in a grinder. 20, 30, 50 grams of dried Kalonji powder was added to 100ml of ethanol separately and kept at room temperature for 72 h. The suspension was filtrated through a sterile filter paper (Whatman-1). The filtrate fractions were stored in screw cap bottles at 4^0C for further study.

***Emblica officinalis & Terminalia Chebula* (AMLA AND HARRO)**

Fruits of amla and Harro were collected from local market of Jabalpur. These two fruit pulp was shade dried and grind into coarse powder separately. The finely coarse powder (20g, 30g, 50g) of amla and Harro was soaked separately in 100ml ethanol for 72 hrs at room temperature. The suspension was then filtered with the help of Whatman-1 filter paper. This ethanol extract was stored in screw cap bottles at 4^0C for further study.

TRIPHALA

Triphala (tri i.e. three and phala i.e. fruits) is a mixture of powder of three fruits namely Harro *(Terminelia chebula*), Bahera (*Terminelia belerica*), and Amla (*Emblica officinalis*). The finely coarse dried powder of three plants was mixed in equal quantity. This dried mixture (20g, 30g, and 50g) was soaked separately in 100ml ethanol for 72 hrs at room temperature. The suspension was filtered with the help of Whaatman-1 filter paper and stored in screw cap bottles at 4^0C.

***Salmonella* Sampling**

A total of 50 urine and stool samples of typhoid patients were collected from different hospitals and some Pathological Labs located in Jabalpur and Narsinghpur district (M.P.)

25 grams of stool sample were taken in 225 ml of buffered peptone water whereas urine sample was collected in sterile vials.

Isolation of *Salmonella* species

The standard procedures (ISO-6579 2002) followed for the isolation of *Salmonella* from urine sample is described as under:-

- Pre-enrichment in non-selective medium (buffered peptone water)
- Selective enrichment in Tetrathionate broth and Rappaport Vassiliadis soy peptone (RVS) broth.

Sub-cultivation on Xylose Lysine Desoxycholate (XLD) agar,

Non-selective pre-enrichment

Buffered peptone water is used as pre-enrichment medium.

Weigh out 25 g stool sample was taken, and put it into a sterile flask and add 225 ml of buffered peptone water to obtain 1 part sample + 9 part buffer. Mix it well and incubate at 37^0 C for 18-24 hr.

Selective enrichment

For selective enrichment two media were used.

(i) Tetrathionate broth and

(ii) Rappaport Vassiliadis soy (RVS) peptone broth

Sub-cultivation on Xylose Lysine Desoxycholate (XLD) agar plates

Sodium desoxycholate is the selective agent and phenol red is the pH indicator. The indicative principle is based on lactose, sucrose and xylose fermentation, H_2S production and lysine decarboxylation. If H_2S is produced from sodium thiosuphate, black FeS (ferrosulfide) will develop. *Salmonella* ferments Xylose, but not lactose and sucrose, decarboxylate lysine and produces H_2S *Salmonella* suspect colonies grow as red colonies with a black center.

Identification of *Salmonella* species

After isolation of colonies from XLD medium, in order to detect *Salmonella* strains, positive, differential and selective biochemical tests such as H_2S production, gas production from glucose, citrate consumption and carbohydrate fermentation (including mannitol, dulcitol, Sorbitol, arabinose and glucose) and negative biochemical test such as urease test, Indole production and carbohydrate fermentation (including sucrose and lactose) were used. For this aim, bacteria were cultured in the nutrient broth medium at 37^0C for 18-24 hr and hence their positive or negative states were distinguished according to phenotypic changes.

For the identification of *Salmonella* spp. biochemical test were performed by using KBO11 Hi media kit.

Preparation of inoculums

- KBO11 cannot be used directly on clinical specimens. The organisms to be identified have to be first isolated and purified. Only pure cultures should be used.
- Isolate the organism to be identified on a common medium like Nutrient broth. Pick up a single well isolated colony and inoculate in 5 ml Nutrient broth and incubate at 37^0 C for 4-6 hour until the inoculum turbidity is $\geq$ 0.1 OD at 620 nm.

Candida albicans

Candida albicans IHEM 22861 were procured from Fungal Disease Diagnostic & Research Center, 1570, Near M.H Hospital, behind Chanchala Bai College Wright Town Jabalpur (M.P). It was sub cultured in Saboruad Dextrose Agar (Dextrose 40mg, Peptone10mg, Agar agar 20mg, Distilled Water 100ml).

Antimicrobial Testing

Agar Well Diffusion Method: The antibacterial activity of different plant extracts (ethanolic extracts of *Azadirachta indica, Emblica officinalis, Terminelia chebula, Nigella sativa* and Triphala) was evaluated by agar well diffusion method (NCCLS, 2001; Okunji, 1900). The inoculums were prepared from bacterial and fungal culture in said medium. About 15-20 ml of Nutrient Agar Medium was poured in the sterilized Petri-dish and allows solidifying. Bacterial strain was spread over the medium on separate petri-dishes. Like wise fungal strain was spread over SDA medium. Wells of extracts 6mm in diameter and about 2 cm apart punctured in the culture medium using sterile Cork borers. About 100 µl of each ethanolic plant extracts was added to the wells. Plates were incubated at 37°C for 24 hours. Antibacterial activities were evaluated by measuring inhibition zone diameters. For each bacterial and fungal strain, control was maintained in which pure solvents were used instead of the extract.

RESULTS & DISCUSSION

Fifty *Salmonella* spp. were recovered from different clinical specimens like blood, urine and stool. These clinical samples were collected from different hospitals and some Pathological Labs located in Jabalpur and Narsinghpur district, Madhya Pradesh. Out of fifty samples seventeen samples were of *Salmonella* spp. As confirmed biochemical tests using KBO11 HiSalmonella kit.12 tests were performed by kit method as shown in table 4.1. Different serovars represent different colors and on the basis of color change, serovars of *Salmonella*

were identified according to the following interpretation chart provided with the kit. Serovars were marked as EBL/A1, EBL/A17, EBL/ A2 was taken as a test bacterium in present study
A total of 5 selected medicinal plants (*Azadirachta indica, Embilica officinalis, Terminalia chebula, Terminalia belerica, Nigela sativa*) have been tested for the antibacterial activity. The tested plants showed negative as well as positive activities against the test bacteria. Though, the response is not uniform, most of plants showed bioactivity against the bacterial strain used in this study (Table 4.2).

In the present study, ethanolic extract of 3 plants (Kalonji, Harro and Trifla) showed activity against gram-negative bacteria *S. typhimurium*. However, the growth of *Candida albicans* was controlled by ethanolic extract of Neem, Kalonji, Harro and Triphla, at a higher concentration which indicated that they could inhibit the activity of fungi at a higher concentration, which can cause diarrhoea and dysentery. Neem has been reported as one of the popular but over exploited medicinal plants in the study area. The rhizome powder of this plant was mainly used to control diarrhoea besides other uses. Seeds of *Nigella sativa* (Kalonji) have been frequently used in folk medicine for several medicinal purposes including to treat cough, asthma, diarrhea, fever, common cold, headache, rheumatic disease, stomach disorders, dyslipidaemia, and infections and to expel worms from the intestine. It is also effective for scorpion and spider strings and bites of snake, cat and dog. Seeds of *N. sativa* have also hypoglycemic, antioxidant, antihistaminic and anti-inflammatory effects. Recently, antibacterial, antiviral, antifungal, antiparasitic, antiprotozoal, and anticancer properties of seeds of *N. sativa* have also been established. Nigellon, thymohydroquinone (THQ), thymoquinone and thymol (THY) are its active ingredients which are responsible for its therapeutic effects. *N. sativa* also contains carbohydrates, fats, vitamins, mineral elements, essential amino acids and proteins. The seeds of *N. sativa* can be poisonous to man in high doses (Saeed and Khan, 2010). Similarly, Harro was most common and one of the widely used medicinal herbs of the area; its fruits were used in stomach problem and as popular household spices. It also showed broad spectrum activity against gram positive and gram negative bacteria (Kannan *et al*., 2009). It was expected that Amla would show activity against *Salmonella* because local users had reported that the flower of this plant was very effective to control dysentery with blood. Sometimes plants may give effective results only when combined with others, as *P. emblica* is one of the best constituents of a well known Ayurvedic drug 'Triphala' i.e. combination of *P. emblica, T. chebula* and *Terminalia. bellirica* in equal proportion is used to relief from stomach disorder.

All the extracts showed varying degrees of antibacterial and antifungal activity against the tested organisms (Table 4.2 and 4.3) with some plant extracts showing strong antimicrobial activity with zones of inhibition between 3.2 to 14.5 mm. *Kalonji* extract showed strong antimicrobial activity against *Salmonella* and *C. albicans* with inhibition zones, 12.00mm and 14.5 mm. respectively.

Biochemical Test Results of *Salmonella* Serovars

TABLE: 4.1

Name of the test	**EBL/A$_1$**	**EBL/A$_2$**	**EBL/A$_3$**	**EBL/A$_4$**	**EBL/A$_5$**	**EBL/A$_6$**	**EBL/A$_7$**	**EBL/A$_8$**
MR	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
H_2S Production	+	+	+	+	+	+	+	+
Citrate	+	-	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+
ONPG	-	-	-	-	-	V	+	-
Lactose	-	-	-	-	-	-	-	-
Arabinose	+	-	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+
Dulcitol	+	-	-	-	-	+	-	-
Name of Serovars	*S. cholerasuis*	*S. typhi*	*S. houtenae*	*S. houtenae*	*S. houtenae*	*S. salmae*	*S. bongoi*	*S. houtanae*

TABLE: 4.1 continued….

Name of the test	**EBL/A$_9$**	**EBL/A$_{10}$**	**EBL/A$_{11}$**	**EBL/A$_{12}$**	**EBL/A$_{13}$**	**EBL/A$_{14}$**	**EBL/A$_{15}$**	**EBL/A$_{16}$**	**EBL/A$_{17}$**
MR	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-
H_2S Production	+	+	+	+	+	+	+	+	+
Citrate	+	V	+	+	-	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+
ONPG	-	V	-	+	-	+	-	+	+

Lactose	-	V	-	V	-	V	-	V	-
Arabinose	+	+	+	+	-	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Sorbitol	+	-	+	+	+	+	+	+	+
Dulcitol	-	V	V	-	-	-	V	-	+
Name of Serovars	*S. houtenae*	*S. indica*	*S. typhimurium*	*S. arizonae*	*S. typhi*	*S. diarizonae*	*S. typhimurium*	*S. arizonae*	*S. bongori*

+ = positive, - = negative, V = variable

Table 4.2 Antibacterial Activity of Ethanolic Extract of Medicinal Plants on *Salmonella*

S.No.	Name of Plants	Average Zone Of Inhibition			
		Salmonella	20g*	30g*	50g*
1	*Neem*		*nil*	*nil*	*Nil*
2	*Kalonji*		*7.7mm*	*9.2mm*	*12mm*
3	*Harro*		*4.1mm*	*6.4mm*	*9.2mm*
4	*Amla*		*nil*	*nil*	*Nil*
5	*Trifla*		*5.7mm*	*8.3mm*	*11.7mm*

*Note: 100µl of each concentration (20g 30g, & 50g) were used for antimicrobial activity

Table 4.3. Antibacterial Activity of Ethanolic Extract of Medicinal Plants on *C.Albicanes*

S.No.	Name of Plants	Average Zone Of Inhibition			
		C.Albicanes	20g*	30g*	50g*
1	*Neem*		*5.2mm*	*7.1mm*	*10mm*
2	*Kalonji*		*8.7mm*	*9.9mm*	*12mm*
3	*Harro*		*8.1mm*	*9.4mm*	*14mm*
4	*Amla*		*nil*	*nil*	*Nil*
5	*Trifla*		*5.7mm*	*8.3mm*	*11.7mm*

*Note: 100µl of each concentration (20g 30g, & 50g) were used for antimicrobial activity.

The ethanolic extract of majority of the plants exhibited higher antimicrobial activity. This credit to ethanol extraction was supposed to be because ethanol is an organic solvent and will

dissolve organic compounds better than aqueous extract and also liberate the active component required for antibacterial activity. It was clear from this study that the solvent of extraction and method of extraction affected the degree of antimicrobial activity. Other factors such as the environmental and climatic conditions of the plants also affected the degree of antimicrobial activity. Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional healers use primarily water as the solvent but in the present study it is found that plant extract in organic solvent provided more consistent antimicrobial activity compared to those extracted in water. Many substances may be antimicrobial, but only a few of them will be potential therapeutic agents for the simple reason that mammalian cells are more sensitive to chemical inhibition than microbial cells (Sivakumar *et al*., 2006). Moreover, emphasized the need for toxicity testing of drugs derived from medicinal plants because the crude products obtained from such cheaper sources are often associated with a large number of compounds that have discomforting abilities (Ramdas *et al*., 2006). Hence the herbal drugs have to be subjected to extensive pharmacological, toxicological and clinical tests to conform the prescribed status. Thus the ethno botanical approach will be like a search for molecular diversity subjecting a wide variety of new molecules from plant sources and testing them with as many different tests as possible. The present study has shown a spectrum of antibacterial activities, which provides a support to some traditional uses of these few medicinal plants. But the effective biomolecules which act as antibacterial have to be identified isolated and subjected to extensive scientific and pharmacological screening that can be used as sources for new drugs.

These findings support the traditional knowledge of local users and it is a preliminary scientific validation for the use of these plants for antibacterial activity. To promote proper sustainable use of such plant resources, awareness of local communities should be enhanced incorporating the traditional knowledge with scientific findings.

CONCLUSSION

Plant based antimicrobial compounds have enormous therapeutically potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials.

Neem, Harro *Kalonji* and Trifla exhibited active antimicrobial activity against the Enterobacteriaceae tested that is, *Salmonella typhimurium* and Fungi *Candida Albicans.*

Kalonji (14.5mm) showed maximum activity against *Salmonella typhimurium* followed by Trifala (10.6mm) and Harro (9.6mm) while in case of C. albicans, Trifala (13.7mm) showed maximum inhibitory activity followed by Kalonji (12mm), Harro (9.2mm) and Neem (5.1mm).

Based on the result obtained in this study, the herbal plant extracts could be much more relevant for use as antimicrobial agent. Synergistic effect experienced in combination of some plant sources for use as antimicrobial agent described by Farnsworths *et al* (1966), works effectively in this study also. The Trifala showed 10.6 mm zone of inhibition against *Salmonella typhimurium* as compared to Harro (9.6mm) whereas in case of *Candida Albicans*, Trifla showed (13.7mm) more zone of inhibition as compared to Harro (9.2mm). Thus, the use of this combination of plant source is recommended for use, combined usage practiced among the indigenes. Apart from combination of plant extracts like Trifala, some individual plants like Kalonji have also shown significant antimicrobial activity against *S. typhi*. Future research on this study can however concentrate on optimizing clinically the chemotherapeutic processes, treatment and control of these common food-borne enteric infections like typhoid in Madhya Pradesh and other parts of the country where the plants can be easily cultivated or otherwise commercialized for pharmaceutical purpose.

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