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# International Journal of Nutrition and Metabolism

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Full Length Research Paper

# Antioxidant activities and phenolic contents of three mushroom species, *Lentinus squarrosulus* Mont., *Volvariella esculenta* (Masse) Singer and *Pleurocybella porrigens* (Pers.) Singer

Israel O. Okoro

Department of Biochemistry, Delta State University, Abraka, Nigeria. E-mail: israelik@yahoo.com.

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The antioxidant properties of three mushroom samples, namely, *Lentinus squarrosulus*, *Volvariella esculenta* and *Pleurocybella porrigens* were investigated. The antioxidant activities, 2, 2-diphenylpicrylhydrazyl (DPPH) radical scavenging ability, reducing powers, amount of total phenolic compounds and flavonoid concentration of the extracts were determined. The mushroom samples showed differences in values for all the five parameters. *L. squarrosulus* had the highest antioxidant activity of  $40.54 \pm 1.50$ , flavonoid concentration ( $61.93 \pm 2.93$ ), total phenolic content ( $392.68 \pm 33.77$ ) and reducing power activity ( $281.15 \pm 8.13$ ), while *P. porrigens* had the least values for these parameters. There were statistically significant correlations between reducing power and amount of total phenolic compounds in all three mushroom extracts. The highest DPPH scavenging ability was shown by *P. porrigens* ( $63.37 \pm 2.89$ ) and the least value of this parameter was shown by *V. esculenta* ( $48.88 \pm 1.35$ ). The mushroom samples showed significant difference ( $p < 0.05$ ) in all the parameters, except for total phenolic compounds concentration ( $p > 0.05$ ). All the three mushroom samples exhibited effective antioxidant properties which contribute to their medicinal and health values.

**Key words:** *Lentinus squarrosulus*, *Volvariella esculenta*, *Pleurocybella porrigens*, mushroom, antioxidant, phenolic content.

## INTRODUCTION

Mushrooms represent one of the world's greatest untapped resources of nutritious food. Cultivation of saprophytic edible mushrooms may be the only currently economical biotechnology for lignocellulose organic waste recycling that combines the production of protein rich food with the reduction of environmental pollution (Obodai et al., 2003). Mushrooms are rich in protein, minerals and vitamins, and they contain an abundance of essential amino acids (Sadler, 2003). Therefore, mushrooms can be a good supplement to cereals (Chang and Buswell, 1996). Mushrooms can be saprophytic or parasitic. They include members of the basidiomycota and some members of the ascomycota. They consist of two main parts, the mycelium and the fruity body (sporocarp).

The arising awareness of the relationship between

diet and diseases has evolved the concept of "functional foods" and the development of a new scientific discipline, Functional Food Science (Sadler and Saltmarsh, 1998). A food may be considered to be functional if it contains a food component (whether a nutrient or not) which affects one or more identified functions in the body in a positive manner, which are in different name forms, e.g. dietary supplements, nutraceuticals, medicinal foods, vita foods, pharma foods, phytochemicals, mycochemicals and foods for specific health uses (Hasler, 1996). The scientific community, in searching for new therapeutic alternatives, has studied many kinds of mushrooms and has found variable therapeutic activity, such as, anticarcinogenic, anti-inflammatory, immunosuppressor and antibiotic, among others (Asfors and Ley, 1993; Longvah and Deosthale, 1998). It has been known for

many years that selected mushrooms of higher basidiomycetes origin are effective against certain cancer types, and this has stirred a growing interest in such mushrooms from industry, the media and the scientific community (Wasser, 2002). Medicinal mushrooms have an established history of use in traditional oriental therapies. Mushrooms have been used for many years in oriental culture as tea and nutritional food, and because of their special fragrance and texture (Manzi et al., 1999).

The human body suffers several negative effects, such as, cancer, stroke, ageing, etc., that could be attributed to either internal or external factors that induce the production of free radicals (Oboh and Akindahunsi, 2004; Oboh, 2005).

Oxidative stress occurs when the production of harmful molecules called free radicals is beyond the protective capability of the antioxidant defences (Alia et al., 2003). Free radicals are chemically active atoms or molecular fragments that have a charge due to an excess or deficient number of electrons. Examples of free radicals are the super oxide anion, hydroxyl radical, and transition metals, such as iron and copper, nitric acid and ozone (Alia et al., 2003). Free radicals containing oxygen known as reactive oxygen species (ROS) are the most biologically significant free radicals. ROS include the radicals super oxide and hydroxyl radical, plus derivatives of oxygen that do not contain unpaired electrons, such as, hydrogen peroxide, singlet oxygen and hypochlorous acid. Free radicals are highly unstable, because they have one or more unpaired electrons (Alia et al., 2003). They scavenge in the body to grab or donate electrons; thereby, damaging cells, proteins and DNA (genetic materials). The same oxidative process also causes oil to become rancid, peeled apples to turn brown and iron to rust (Alia et al., 2003). Normally, bonds split in ways that leave a molecule with an odd, unpaired electron, but when weak bonds split, free radicals are formed (Alia et al., 2003). Antioxidants are substances that are capable of counteracting the damaging, but the normal effects of the physiological process of oxidation in animal tissue. Antioxidants are nutrients (vitamins and minerals) as well as enzymes (protein in the body that assists in chemical reactions) (Sun et al., 2002). They are believed to play the role of preventing the development of such chronic diseases as cancer, heart disease, stroke, rheumatoid arthritis and cataracts (Chu et al., 2002).

Almost all organisms are well protected against free radical damage by enzymes, such as, superoxide dismutase and catalase, or compounds, such as, ascorbic acid, tocopherols and glutathione (Mau et al., 2002). When the mechanism of antioxidant protection becomes unbalanced by factors, such as, aging, deterioration of physiological functions may occur resulting in diseases and accelerated aging. However, the antioxidants present in human diet are of great interest as possible protective agents to help the human bodies reduce oxidative damage. Researchers have

reported the antimicrobial and antioxidative activities of several mushrooms (Lee et al., 1999; Gao et al., 2005; Turkoglu et al., 2006; Iwalokun et al., 2007), amongst other benefits of mushrooms. Therefore, this study is aimed at evaluating the antioxidant potential of three edible mushroom samples, namely, *Lentinus squarrosulus*, *Volvariella esculenta* and *Pleurocybella porrigens*.

## MATERIALS AND METHODS

### Mushrooms

Mushroom samples were collected from farm lands in Abraka, Delta State, Nigeria. They were identified by a mycologist, Mr. A. O. Oghenekaro, at the Department of Botany, University of Benin, Benin City, Edo State, Nigeria. Further analyses of the samples were done at the laboratory of Department of Biochemistry, Delta State University, Abraka. Fresh mushroom samples (150 g) were air dried in an oven at 40°C before analysis. Dried mushroom sample (50 g) was extracted by stirring with 500 ml of ethanol at 30°C at 150 rpm for 24 h and filtering was done through Whatman No. 4 filter paper. The residue was extracted with two additional 500 ml of ethanol as described earlier. The combined ethanolic extract were then rotary evaporated at 40°C to dryness, redissolved in ethanol to a concentration of 10 mg/ml and stored at 4°C for further use.

### Antioxidant activity

The antioxidant activity was determined by ammonium thiocyanate assay (Lee et al., 2002). 500 µl of extract, 200 µl of diluted linoleic acid (25 mg/ml 99% ethanol) and 400 µl of 0.5 M phosphate buffer (pH 4) was mixed and incubated at 40°C for 15 min. Aliquot (100 µl) from the reaction mixture was mixed with reaction solution containing 3 ml of 70% ethanol, 100 µl of ammonium thiocyanate (300 mg/ml distilled water) and 100 µl of ferrous chloride (2.45 mg/ml in 3.5% hydrochloric acid). The final reaction solution was mixed and incubated at room temperature for 3 min.

Absorbance was measured at 500 nm. Linoleic acid emulsions without extract served as control. Inhibition of linoleic acid oxidation was calculated by using the following formula:

$$\text{Inhibition (\%)} = \left[ \frac{(\text{control OD} - \text{sample OD})}{\text{control OD}} \right] \times 100.$$

OD = Optical density

### DPPH assay

Hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2, 2-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay was done according to Blois (1958), with a slight modification. 500 µl of extract solution was mixed with 1 ml of 0.1 mM DPPH in ethanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4) was added. The solution was incubated at 37°C for 30 min and reduction of DPPH free radicals was measured by reading the absorbance at 517 nm. Control was maintained. Ascorbic acid solution was used for comparison. This activity was given as percentage DPPH scavenging and calculated according to the following equation:

$$\text{DPPH scavenging (\%)} = \left[ \frac{(\text{control OD} - \text{sample OD})}{(\text{control OD})} \right] \times 100$$

### Determination of total phenolic compounds

The amount of total phenolic compound content of the extracts was determined by the method described by Singleton et al. (1999). 500  $\mu$ l of extract was transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by addition of distilled water. 1 ml of Folin-Ciocalteu reactive solution was added and incubated at room temperature for 3 min. 3 ml of 2% sodium carbonate solution was added to the mixture and was shaken on a shaker for 2 h at room temperature. The absorbance was measured at 760 nm.

Gallic acid was used as the standard for a calibration curve. The phenolic compound content was expressed as gallic acid equivalent.

### Determination of total flavonoid concentration

Flavonoid concentration was determined as follows: Mushroom ethanolic extracts solution (1 ml) was diluted with 4.3 ml of 80% aqueous ethanol and test tubes was added to 0.1 ml of 10% aluminum nitrate and 0.1 ml of 1 M aqueous potassium acetate. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as standard (Park et al., 1997).

### Test for reducing power

Phosphomolybdenum (PMo) assay according to Prieto et al. (1998) with slight modification was used to estimate the capability of the samples to reduce transition metal ions. The reagent solution contained ammonium molybdate (4 mM), sodium phosphate (28 mM), and sulfuric acid (600 mM) mixed with the samples diluted in ethanol. The samples were incubated at 90°C for 90 min, cooled down to room temperature, and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. The reducing capacity of the extracts was calculated using the following equation:

$$\text{ABS final} = \text{ABS sample} - \text{ABS blank} - \text{ABS extract}$$

where ABS extract = absorbance of sample where molybdate solution was replaced by water; ABS blank = absorbance of blank containing methanol (400  $\mu$ l) instead of extract sample.

For reference, the appropriate solutions of ascorbic acid have been used, and the reducing capacity of the analyzed extract was expressed as the ascorbic acid equivalent (AAE) per gram of sample dry weight.

## RESULTS

The reducing power, total phenolic compounds and flavonoid concentration of ethanolic extracts of *L. squarrosulus*, *V. esculenta* and *P. porrigens* were determined, and the results are as shown in Table 1. The reducing power PMo test shows that *L. squarrosulus* had the highest value (281.15 $\pm$ 8.13), and the least value was displayed by *N. porringes*. Comparatively, all the three mushrooms significantly differed ( $p < 0.05$ ), when compared against one another. The total phenolic compounds values in Table 1 shows that the highest and least value were displayed by *L. squarrosulus* and *P. porrigens*, respectively, but there was no statistical difference ( $p > 0.05$ ), when compared against one another.

On the other hand, there was significant difference ( $p < 0.05$ ), when the flavonoid concentration values were compared.

The result of the DPPH scavenging ability and antioxidant activity (Table 2) showed that *P. porrigens* and *L. squarrosulus* had the highest and least value for the DPPH scavenging ability while, the lowest value for antioxidant activity was shown by *P. porrigens*, and the highest value displayed was by *P. squarrosulus*, whereas, there was significant difference ( $p < 0.05$ ), when all the three mushrooms were compared for the antioxidant activity. This was however not seen in the percentage DPPH scavenging ability, where the comparative value at  $p < 0.05$  was not significant when *L. squarrosulus* was compared with *V. esculenta*, but there was statistical significant difference ( $p < 0.05$ ), when *L. squarrosulus* was compared with *P. porrigens*, and when *V. esculenta* was compared with *P. porrigens*.

## DISCUSSION

Mushrooms have been shown to contain vast varieties of biologically active substances with immunostimulatory, anti-cancer and antioxidant properties (Silva, 2004). Mushrooms from various researches have demonstrated several medicinal importances, which is attributed to the presence of many bioactive components which may include polysaccharides, proteins, vitamins and aromatic compounds like polyphenols (flavonoids, tannins, saponins, alkaloids, etc). All the extracts of the three mushrooms (*L. squarrosulus*, *V. esculenta* and *P. porringes*) exhibited antioxidant properties. The results of this study, as shown in Tables 1 and 2 demonstrate a good correlation between total content of phenolic compounds and the antioxidant activity of the three mushrooms investigated, and this is in agreement with previous findings (Cai et al., 2004; Beta et al., 2005; Othman et al., 2007; Tawaha et al., 2007).

The most effective free radical scavenging activity was shown by the extract of *P. porringes* (63.37 $\pm$ 2.89), the least activity was observed in *V. esculenta* (48.88 $\pm$ 1.35) (Table 2). The most effective antioxidant potential was shown by the extract of *L. squarrosulus* (40.54 $\pm$ 1.50), and the least activity was shown by *P. porringes* (20.06 $\pm$ 0.60).

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). ROS and associated free radicals have been implicated in the etiology of various human diseases, including inflammation, metabolic disorders, cellular aging and atherosclerosis, heart disease, stroke, diabetes mellitus, cancer, malaria, rheumatoid arthritis and HIV/AIDS (Alho and Leinonen, 1999; Odukoya et al., 2005). Therefore, radical scavengers give promising indications of new therapeutic approaches

**Table 1.** Determination of reducing power, total phenolic compounds and flavonoid concentration of aqueous extract of *L. squarrosulus*, *V. esculenta* and *P. porrigens*.

Parameter	<i>L. squarrosulus</i>	<i>V. esculenta</i>	<i>P. porrigens</i>
Reducing power by PMo test (AAE mg/g) $\pm$ SEM	281.15 $\pm$ 8.13*	211.60 $\pm$ 8.64*	137.07 $\pm$ 4.58*
Total phenolic compounds ( $\mu$ g GAE/100g)	392.68 $\pm$ 33.77	295.71 $\pm$ 4.46	289.71 $\pm$ 6.53
Flavonoid concentration (mg/g)	61.93 $\pm$ 2.93*	32.17 $\pm$ 1.64*	20.70 $\pm$ 1.18*

All values are expressed as mean  $\pm$  standard error of mean (SEM) (n=3). \*, Differ statistically at p<0.05; n = number of determinations; GAE, gallic acid equivalents; AAE, ascorbic acid equivalent.

**Table 2.** Determination of DPPH scavenging ability and antioxidant activity of *L. squarrosulus*, *V. esculenta* and *P. porrigens*.

Parameter	<i>L. squarrosulus</i>	<i>V. esculenta</i>	<i>P. porrigens</i>
DPPH scavenging ability (%)	53.04 $\pm$ 2.63	48.88 $\pm$ 1.35*	63.37 $\pm$ 2.89*
Antioxidant activity (ammonium thiocyanate assay % of mushrooms)	40.54 $\pm$ 1.50*	21.18 $\pm$ 1.26*	20.06 $\pm$ 0.60

Data are expressed as mean  $\pm$  standard error of mean (SEM) for three determinations. \*, Differ statistically (p<0.05).

(Cho et al., 2003).

The significance of antioxidant evaluation of these three mushrooms extracts lie in the structural requirement of the chemical constituents that could be linked to flavonoids, which have been found present in the all the three extracts evaluated (Table 1). Many flavonoids have shown strong antioxidant properties (Raj and Shalini, 1999). The capacity of plant extracts to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators or free radical scavengers indicates their potential therapeutic properties for treating diseases related to free radical reactions. The results of this study showed that the reducing power activity, amount of total polyphenols and concentration were higher in the extract of *L. squarrosulus* than the other two mushrooms (Table 1). There were statistically significant correlations between reducing power and amount of total phenolic compounds in all three mushroom extracts, which is in agreement with the findings of other researchers. Yildirim et al. (2001) have suggested that there may be relationship between phenolic compounds and reducing powers. The reducing capacity of compounds could serve as indicator of potential antioxidant properties (Meir et al., 1995).

The presence of phenolic compounds might be the reason for reducing power. Phenolic compounds are also thought to be capable of regenerating endogenous  $\alpha$ -tocopherol, in the phospholipid bilayer of lipoprotein particles, back to its active antioxidant form. They are also known to inhibit various types of oxidizing enzymes. These potential mechanisms of antioxidant action make the diverse group of phenolic compounds an interesting target in the search for healthbeneficial phytochemicals (Halliwell and Gutteridge, 1989; Hall and Cuppett, 1997). The key role of phenolic compounds as scavengers of free radicals is emphasised in several reports (Komali et al., 1999; Moller et al., 1999). Phenols are important

components of plants. They are reported to eliminate radicals due to their hydroxyl groups (Hatano et al., 1989), and they contribute directly to antioxidant effect of system (Duh et al., 1999). Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen et al., 1993; Gulcin et al., 2003). The phenolic compounds may contribute directly to antioxidative action (Duh et al., 1999). It has been suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables (Tanaka et al., 1998).

Like phenol compounds, the contribution of flavonoids to antioxidant activity is known. It has been reported that butylated hydroxytoluene (BHT) I3, I18-biapigenin and hypericine which have the structure of biflavonoid have a very high antioxidant effect. This effect was proposed to stem from hydroxyl groups in the structure of the flavonoids (Cakir et al., 2003). Therefore, the mushrooms extracts investigated in this study could be said to compete favourably with butylated hydroxyanisole (BHA) and  $\alpha$ -tocopherol in  $\beta$ -caroten-linoleic acid system used to determine the antioxidant capacity.

In conclusion, the results of this study indicate that the mushrooms investigated are enriched with antioxidant potential and would be the choice of selection for commercialization and are therefore recommended for consumption, since they have health benefits.

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Full Length Research Paper

# Nutritional status, bacterial vaginosis and cervical colonization in women living in an urban slum in India

Rajkumar Hemalatha\*, Baru Anantha Ramalaxmi, Gummuluri Krishna Swetha, Dasari Madusudhan Rao, Sesha Charyulu, and Dinesh Kumar

National Institute of Nutrition, Jamaio-Osmania PO, Hyderabad – 500007, Andhra Pradesh, India.

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The prevalence of bacterial vaginosis and cervical colonization, and association of bacterial vaginosis with serum nutrients were determined in women living in slum areas of Hyderabad, India. Bacterial vaginosis was diagnosed based on Nugent's score. Cervical infections with human papilloma virus, herpes simplex virus type 2, *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* were determined by PCR. Of the 260 women who participated in the study, 31% (81) had bacterial vaginosis and 48.8% (127) had intermediate flora based on Nugent's score. Only 184 vaginal samples were processed for candidiasis, of which 66 showed *Candida albicans*, accounting for a prevalence of 36.0%. PCR analysis of cervical swabs obtained from 50 women with acute cervicitis showed the following trend of prevalence of various organisms: 30% *U. urealyticum*, 10% *M. hominis*, 2% herpes simplex virus and Human papilloma virus, while *C. trachomatis* and *N. gonorrhoea* were not detected in any. In the 50 women without cervicitis, 6 (12%) had human papilloma virus, while other organisms were not detected. All the women with cervical colonization (*U. urealyticum*, *M. hominis* and herpes simplex virus type 2) and 6 of 7 women with human papilloma virus had bacterial vaginosis or intermediate flora. Thirty percent of the women were undernourished (body mass index <18.5), while all the women in the study were anemic (hemoglobin <12 g/dl). More than 50% of the women in the study had low serum iron, while more than 90% had low serum zinc levels. But vitamin A deficiency was prevalent in 3% of the study subjects. Low concentrations of serum iron, zinc and vitamin A were significantly associated with bacterial vaginosis.

**Key words:** Bacterial vaginosis, cervicitis, *Ureaplasma urealyticum*, *Mycoplasma hominis*, vitamin A, iron, zinc.

## INTRODUCTION

Bacterial vaginosis (BV) is the most common cause of lower genital tract infections in women of reproductive age group and is associated with increased susceptibility to sexually transmitted infections, preterm deliveries and HIV infection (Angela, 2008; Sabina et al., 2002; Allsworth and Peipert, 2007; Sewankambo et al., 1997; Hillier, 1998). It is characterized by a disturbance in

normal vaginal flora with loss of lactobacilli and increasing numbers of anaerobes and gram negative rods. BV is often asymptomatic and relapses are frequent (Jyoti et al., 2010). Several factors such as race, smoking, chronic stress, vaginal douching and contraceptive use are associated with BV (Nansel et al., 2006), yet the etiology is not very well understood. In recent years, nutrition is also being hypothesized to be another putative risk for BV (Verstraelen et al., 2005; Beth et al., 2007). Subclinical iron deficiency and low levels of serum vitamin D have been shown to be independently associated with prevalence of BV (Verstraelen et al., 2005; Lisa et al., 2009). Similarly, low dietary intake of micronutrients and high intakes of fat have been associated with BV (Neggert et al., 2007).

\*Corresponding author. E-mail: [rhemalathanin@yahoo.com](mailto:rhemalathanin@yahoo.com).  
Tel: 040-27191297. Fax: 040-27019074.

**Abbreviations:** BV, Bacterial vaginosis; BMI, body mass index; HPV, human papilloma virus; HSV-2, herpes simplex virus type 2.

BV is very common and ranges from 11 to 62% in different populations in India (Neeraja et al., 2009; Patel et al., 2006; Aggarwal et al., 1999; Bang et al., 1989). In a study conducted in Karnataka, India, prevalence of bacterial vaginosis was 20% and another study from Haryana showed 48% prevalence of BV (Rao et al., 2004; Bhalla et al., 2007). Though BV is widely reported and recurrences are common, no information is available on nutritional status of women with BV in India, despite the fact that a sizable proportion of women in India are undernourished (Fred et al., 2009). In the current study, we evaluated BV and cervical colonization and studied the association of BV with serum concentration of nutrients.

## MATERIALS AND METHODS

A cross-sectional community based study was carried out among the population of slum areas of low socioeconomic status in Hyderabad city, India. The study was approved by Institutional Ethical Committee. Households were selected by systematic random sampling method. House-to-house survey was carried out to select women, who were asymptomatic, 20 to 40 years, HIV negative, non-pregnant and living with husband and who were between 8<sup>th</sup> to 10<sup>th</sup> day of menstrual cycle. 300 women fulfilled our eligibility criteria, of which 285 agreed to participate in the study. These 285 women were ferried to the health centers located in the area. In the health centers, demographic and clinical data were collected using a structured questionnaire and after excluding those using oral antibiotics/ contraceptives/ vaginal medication in the last 10 days and those who had sexual intercourse in the last 2 days, we had 262 women. Height and weight were measured to calculate body mass index (BMI) (weight in kg/ height in m<sup>2</sup>). General and gynecological examinations were done to evaluate reproductive health. Vaginal specimens, and cervical swabs were obtained after taking a written consent, but 2 women had bleeding during examination and were excluded. Thus 260 specimens were processed for diagnosing BV based on Nugents' score. All enrolled women denied using douches or tampons and were nonsmokers.

### Gram stain

After pelvic and speculum examination, vaginal smears were collected for wet mount and gram stain. Gram stained smears were scored for gram negative and gram positive bacteria, clue cells, yeast and pus cells (leukocyte counts) to diagnose bacterial vaginosis or vaginitis. Wet mount was prepared to screen for *Trichomonas vaginalis*. BV and intermediate flora were diagnosed based on vaginal evaluation by Nugents' score. Three different bacterial morphotypes - lactobacilli, *Gardnerella*-like species (including *Gardnerella vaginalis*, *Bacteroides* species, *Prevotella* species, and *Porphyromonaz* species), and *Mobilincus* species were quantitatively evaluated according to the Nugent's score method (Nugent et al., 1991). Women with Nugent scores of 0 to 3 were categorized as normal flora. Women with scores of 4 to 6 were classified as intermediate flora and women with Nugent score 7 or more were enrolled as BV.

### Multiplex PCR for cervical pathogens

Cervical swabs from 50 women with cervicitis (>30 WBC/HPF) and 50 women without cervicitis were screened for cervical colonization.

Cervical swabs were processed for human papilloma virus (HPV), herpes simplex virus type 2 (HSV-2), *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum*. Genomic DNA was isolated from the cervical swab samples using Bioserve Genomic DNA isolation kit. The genomic DNA was subjected to multiplex PCR for the detection of HPV, *M. hominis*, *C. trachomatis*, HSV-2, *N. gonorrhoea* and *U. urealyticum*. The PCR products were resolved on a 2% Agarose gel.

### Micronutrients in the serum

For comparing micronutrient status of women with and without BV, blood samples were obtained from 52 women with normal vaginal flora, 52 women with intermediate flora and 80 women with BV. BV cases were age and socioeconomic status matched with controls. A sample size of 50 was decided based on the results of a previous study (Verstraelen et al., 2005). Iron, zinc, calcium, magnesium, and copper were analyzed by atomic absorption spectroscopy (AAS) and vitamin A was determined by high-performance liquid chromatography (HPLC). For iron estimation, serum was treated with one volume of 20% (w/v) trichloro acetic acid (TCA) and heated to ensure release of transferrin bound iron and then centrifuged. The supernatant was diluted with 3 volumes of deionized water and then analyzed by AAS at 248.3 nm. For zinc, one part of the serum sample was diluted in 5 parts of deionized water and mixed well. The supernatant was read at 213.9 nm. Vitamin A was detected at 325 nm using a sensitive ultra violet (UV) detector. Retinyl acetate was used as internal standard to account for processing losses for vitamin A analysis. Serum lipid profile (high density lipoprotein cholesterol (HDL-C) and triglycerides (TG)) were analysed by kits obtained from Biosystems (Barcelona, Spain).

### Statistical analysis

Log-transformed micronutrient mean values were compared between women with BV and without BV by using a Student's t test. Pearson chi-squared test was used to study the association of BV with micronutrient status. To assess the relationship between serum nutrients levels and vaginal flora, Pearson's correlation was done. Logistic regression was used to assess the relation of each nutrient variable to BV. P-values < 0.05 were considered as statistically significant. Statistical analysis was performed using SPSS statistical software (SPSS Inc, Chicago, IL, USA).

## RESULTS

The mean age of the women was 27.9 years. All the women were of low socioeconomic group, semi-literate and apparently normal. Of the 260 women, 31% had BV and 48.8% had intermediate flora based on Nugents' score. Only 184 vaginal samples were processed for candidiasis, which was prevalent in 36.0%. Nearly 33% had acute cervical erosion and 47% had tender fornices on one/both sides (Table 1). On clinical examination, 23 (28%) women with BV and 36 (28%) women with intermediate flora had vaginal discharge, while 11 (21%) women with normal vaginal flora had vaginal discharge.

PCR analysis of cervical swabs obtained from 50 women with acute cervicitis showed 15 (30%) and 5 (10%) women positive for *U. urealyticum* and *M. hominis* respectively. HSV-2 and HPV were prevalent in 2% of the

**Table 1.** Reproductive tract infections (RTI) in women living in slum.

Laboratory-diagnosed RTI	Total Nos.	Number positive	Percentage positive
Bacterial vaginosis (BV)	260	81	31.1
Intermediate vaginal flora	260	127	48.8
Vaginal candidiasis	184	66	36.0
Human papilloma virus	100	7	7
Herpes simplex virus type 2	100	1	1
<i>Mycoplasma hominis</i>	100	5	5
<i>Ureaplasma urealyticum</i>	100	15	15
<i>Chlamydia trachomatis</i>	100	None	0
<i>Neisseria gonorrhoea</i>	100	None	0
Clinically diagnosed RTI			
Vaginal discharge	260	67	26.0
Acute cervical erosion	260	87	33.4
Both fornices tender (PID)	260	58	22.3
Single fornix tender (PID)	260	65	25.0

Nugent's classification was followed for BV and intermediate vaginal flora. Nugent score: 0 to 3 is normal flora, 4 to 6 is intermediate flora and equal to or greater than 7 score is BV. Candida infection was based on gram stain. Human papilloma virus, herpes simplex virus type 2, *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* were detected by polymerase chain reaction (PCR) of samples obtained from cervix.

**Table 2.** Serum nutrients in undernourished (BMI <18.5) women.

Parameter	Less than 18.5 BMI (n=56)	More than or equal to 18.5 BMI (n=128)	Total mean of nutrients	Proportion with deficiency
Hemoglobin (g/dl) (n=175)	11.4 ±0.14	11.7 ±0.09	11.6 ±0.07	2.28% (<9 g/dl)
Calcium (mg/dl) (n=171)	8.74±0.09	9.05±0.11	8.9±0.08	54.5% (<9 mg/dl)
Vitamin A (µg/dl) (n=174)	48.22±3.21	53.80±2.56	52.3±1.66	2.9% (<20 µg/dl)
Zinc (µg/dl) (n=181)	47.25±2.85	48.03±1.74	47.7±1.27	90.6% (<70 µg/dl)
Copper (µg /dl) (n=176)	70.97±3.32	75.45±2.67	78.5±1.99	58.5% (<80 µg/dl)
Iron (µg/dl) (n=184)	68.24±6.78	70.38±4.72	72.6±3.95	49.5% (<60 µg/dl)

Values are mean ± standard error (SE). BMI = body mass index.

women with cervicitis, while *N. gonorrhoea* and *C. trachomatis* were not detected in any (Table 1). In 50 women without cervicitis, 6 (12%) had HPV, while other organisms were not detected. All women with cervical colonization (*U. urealyticum*, *M. hominis* and HSV-2) had BV or intermediate flora and 6 out of 7 HPV positives were associated with BV.

The mean (standard error; SE) BMI was 21.30 (0.387), and 30% of the women had BMI less than 18.5. All the women in the study had hemoglobin less than 12 g/dl. More than 50% of the women in the study had low serum iron, copper and calcium, while low serum zinc was prevalent in 90% of the study subjects (Table 2). However, serum vitamin A was within normal range in 97% of the subjects. Women with low BMI (<18.5) had lower concentrations of serum nutrients (Table 2).

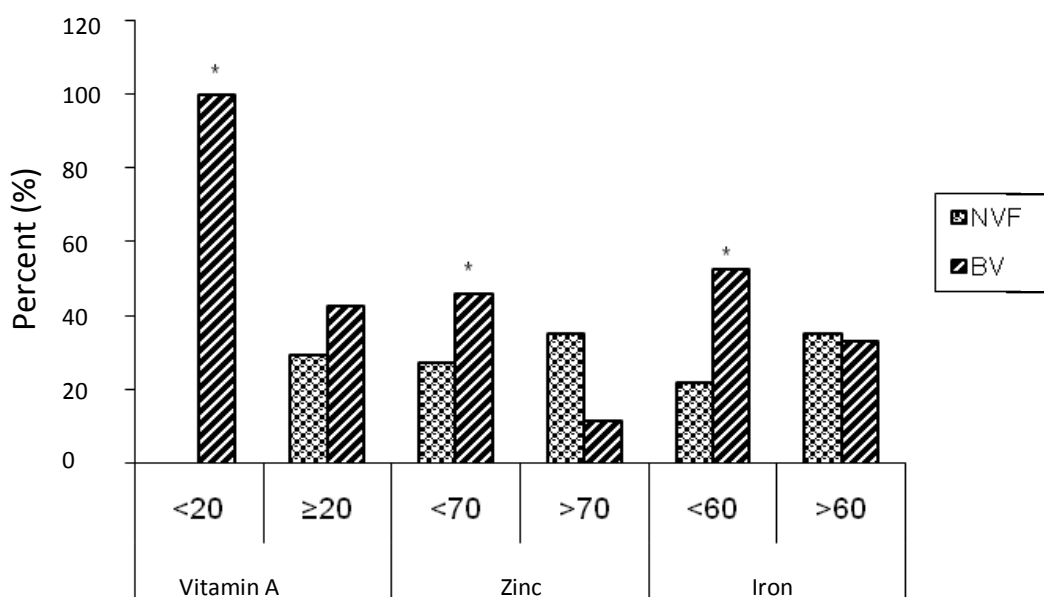
For comparing micronutrient status of women with and without BV, 80 women with BV were taken as case, and 52 women with normal vaginal flora were taken as controls. BV cases were age and socioeconomic status matched with controls. As indicated in Table 3, the mean serum vitamin A, zinc and iron concentrations were significantly ( $P<0.005$ ) lower in the BV compared to the normal vaginal flora and intermediate flora. Similarly, higher proportion of women with vitamin A, zinc and iron deficiencies had significantly ( $p<0.05$ ) higher prevalence of BV (Figure 1). When correlation analysis was done taking Nugent's score as continuous variable, low levels of serum vitamin A and zinc were associated with higher Nugent's score, while serum iron deficiency showed a trend (Table 4). However, logistic regression analysis showed 2 fold higher risk (OR 1.95; 95% confidence



**Table 3.** Mean of serum nutrients in women with BV, intermediate flora and normal vaginal flora.

Parameter	Normal vaginal flora (n=52)	Intermediate flora (n=52)	BV positive (n=80)	P values
Heamoglobin (gm/dl)	11.7±0.14	11.7±0.14	11.5±0.12	>0.05
Calcium (mg/dl)	8.81±0.21	9.05±0.10	8.88±0.07	>0.05
Vitamin A (µg/dl)	57.99±3.42	53.2±2.44	47.95±2.55	0.007
Zinc (µg/dl)	50.61±2.22	52.45±2.6	42.58±1.75	0.021
Copper (µg/dl)	81.98 ± 3.58	77.59±4.20	75.33± 3.67	>0.05
Iron(µg/dl)	87.08±7.5	72.28±5.51	63.03±6.62	0.004
Total cholesterol (mg/dl)	138.95±4.98	124.84±4.51	138.55±5.61	>0.05
Triglyceride (mg/dl)	104.3±7.56	86.44±5.93	96.08±5.55	>0.05
High density lipoprotein (HDL) (mg/dl)	36.24±2.07	33.02±1.11	35.0±1.11	>0.05

Values are mean ± standard error (SE). Mean micronutrient concentrations were compared in women with normal vaginal flora, intermediate flora and BV using a Student's t test.



**Figure 1.** Prevalence (%) of BV in women with micronutrient deficiency. A Pearson chi-squared test was used to study the association of BV with micronutrient status. Y axis depicts percentage prevalence of BV and X axis depicts cutoff values for deficient and normal concentration of nutrients. BV: bacterial vaginosis, NVF: normal vaginal flora. \*P<0.05 BV significantly higher in women with vitamin A, zinc and iron deficiency. Vitamin A, zinc and iron are expressed as µg/dl.

**Table 4.** Correlations of vitamin A, zinc and iron with Nugent's score (BV).

Parameter	R values	P values
Vitamin A	-0.196**	0.009
Zinc	-0.207**	0.005
Iron	-0.138	0.062

\*\* P<0.01. This table depicts correlation analysis with Nugent's score and nutrients as continuous variable. Low concentrations of serum vitamin A, zinc and iron are associated with higher Nugent's score.

interval, 1.01 to 3.75) of BV in women with iron deficiency. Serum copper and calcium concentrations were not associated with BV. Lipid profile such as total cholesterol, triglyceride (TG) and high density lipoprotein (HDL) were similar in women with or without BV (Table 3).

## DISCUSSION

In the current study, on asymptomatic women, not complaining of vaginal discharge, BV was prevalent in

31% and there was modest association between altered vaginal flora and microbial colonization of the cervix. Low serum concentrations of iron, zinc and vitamin A were significantly associated with BV.

Women with cervicitis (>30 WBC/HPF) had *U. urealyticum*, *M. hominis* and HSV-2 colonization, but HPV colonization was not associated with cervicitis. Nevertheless, all cervical colonization (*U. urealyticum*, *M. hominis* and HSV-2) including HPV were associated with BV or intermediate flora. Cervicitis may be caused by infections with *U. urealyticum*, *M. hominis* and sexually transmitted infections, such as chlamydia, gonorrhea and trichomonias (Rodrigues et al., 2011). HPV also causes cervicitis, but not all strains of HPV cause cervicitis. Probably, HPV colonization in women without cervicitis in the current study was due to those strains that do not cause cervicitis.

Most women with BV suffer with multiple recurrences despite antibiotic therapy (Jyoti et al., 2010). Mycoplasma and ureaplasma colonisation have consistently been found in women with BV and have been linked with recurrent BV (Keane et al., 2000; Angela, 2008). Though the current study was not designed to address the issue of recurrent BV and *M. hominis* or *U. urealyticum*, all the women with cervical *M. hominis* and *U. urealyticum* colonization had BV. Few studies in India have reported prevalence of mycoplasma and ureaplasma infections. Our findings on mycoplasma and ureaplasma colonization are similar to Brabin et al. (1998), but lower than that reported in pregnant women (Choudhury et al., 1994); however, none of these studies attempted correlation of BV with these infections. In the subsample of 100 women that we tested, none were positive for *C. trachomatis* or *N. gonorrhoeae* infections. Similarly, a study from Mumbai (India) registered a very low prevalence of *C. trachomatis* (0.5%) and *N. gonorrhoeae* (none). In contrast, Singh et al. (2003) from Delhi (India) reported a very high prevalence of *C. trachomatis* (29%), but the study was on symptomatic patients. *C. trachomatis* or *N. gonorrhoeae* prevalence is low in India but this is not surprising, given the conservative attitudes about extramarital relationships in India.

About one third of the women in the present study had chronic energy deficiency (CED) (BMI < 18.5) and surprisingly, all the study subjects were anemic. Even in a small sample size as this, the study showed a clear correlation of BMI and serum nutrients with lower concentration of all nutrients studied in CED women. More than 60% prevalence of zinc deficiency had been reported by others from India (Kapil et al., 2003; Priyali et al., 2008); comparatively, a higher prevalence was registered in the present study. Similarly, copper, serum iron and calcium levels were low in majority of the study subjects; however, vitamin A was deficient only in 3% of the subjects.

In an earlier study, BV was similar in undernourished (BMI 16.5 to 18.5) and well-nourished women (BMI

>18.5), but was significantly higher in women with severe under-nutrition (BMI <16.5) (Yashodhara et al., 2006). The findings of the current study are in agreement with the observations of the aforementioned study. Correlations of vitamins A and D with reproductive infections have been reported by others (Beth et al., 2007; Belec et al., 2002; Lisa et al., 2009). Association of BV with high fat and low micronutrients intake was reported by Neggers et al. (2007). Low levels of serum micronutrients such as zinc, iron and vitamin A were highly correlated with BV in the current study. As far as our knowledge goes, this is the first study demonstrating low serum zinc and vitamin A in women with BV. Beth et al. (2007) had reported increased risk of HPV infection with low serum zinc concentration, but there are no studies relating zinc with BV. Subclinical iron deficiency has been shown to be independently associated with BV during early pregnancy (Verstraelen et al., 2005). In the current study, logistic regression showed two fold higher risk of BV with iron deficiency in non-pregnant women.

Micronutrients such as vitamin A, zinc and iron play an integral part in both cell mediated and humoral immunity (Bhaskaram, 1997). Vitamin A plays a key role in maintaining the integrity of all the epithelial surfaces in the body, such as the skin, the lining of the respiratory tracts, digestive tracts and the vagina. In vitamin A deficiency, squamous and keratinized epithelial cells replace mucus-secreting cells, thus making these surfaces vulnerable to external environment and foreign invaders. Iron and zinc are important minerals for several enzymes and metabolic pathways. Iron and zinc deficiencies impair cell mediated immunity, delayed hypersensitivity and leukocyte functions (Bhaskaram, 1997). The ability of leukocytes to kill ingested bacteria is impaired. Not much is known about recurrences and relapses of BV, though local vaginal immunity is thought to play an important role in the development of BV (Yashodhara et al., 2006). Micronutrient deficiencies can affect vaginal immune function and may contribute to recurrences and relapses of BV, however, it needs to be explored in a prospective cohort study.

The major weakness of the study is the cross sectional nature, however, this study has been useful in identifying serum nutrients association with BV that can be more rigorously studied using a prospective cohort design. Iron deficiency indicators such as serum transferrin receptors and ferritin were not determined. Serum transferrin receptors and ferritin are important when serum iron levels are >60 µg/dl. However, when serum iron levels are <60 µg/dl, there is no need for additional indicators of iron deficiency. The time of sample collection was a definite strength, because all the women in the current study were recruited during estrogen phase; thus minimizing the hormonal influence on vaginal flora.

BV is the most common vaginal infection, and its impact on the health of women is substantial. Nevertheless, its treatment and prevention remain

difficult. Women with BV suffer with multiple recurrences despite antibiotic therapy. There is a great need for a prospective study that identifies the risk factors for BV and recurrence of BV in India. Research is also warranted in finding good preventive measures and dietary interventions if any for BV.

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