International Journal of Pharmacy

Journal Homepage: http://www.pharmascholars.com

**Research Article** **CODEN: IJPNL6**

# PREVALENCE OF MULTI-DRUG RESISTANT BACTERIA IN (*PIPER BETEL* L) LEAF WASHED-WATER OF THE ROAD SIDE PAN STALL IN NORTH BENGAL

Swapan Kumar Chowdhury

Deptt. of Botany, Sreegopal Banerjee College, Bagati, Mogra, Hooghly, Pincode-712148, West Bengal, India

***Corresponding author e-mail:** chowdhuryswapankr3@gmail.com

**ABSTRACT**

The current study was aimed to evaluate the prevalence of multi-drug resistant pathogenic bacteria in Betel (*Piper betel* L) leaf washed-water of the road side pan stall in North Bengal. This study deals with the determining of coliform counts through MPN test, total heterotrophic load of bacteria, isolation and identification of the Bacteria through plating on different selective medium and determination of multiple antibiotic resistance Bacteria. MPN test shows that sample -2, sample-5 and sample-6 gives positive results. Total heterotrophic load of sample 1 was $5.85 \times 10^{5}$ colony forming unit per milliliter (cfu/ml), sample 2 was $6.64 \times 10^{5}$ cfu/ml, sample 3 was $27.8 \times 10^{5}$ cfu/ml, sample 4 was $12.1 \times 10^{5}$ cfu/ml, and sample 5 was $23.2 \times 10^{5}$ cfu/ml. Many antibiotic resistant pathogenic bacteria are found in leaf wash-water. Significance and impact of this study is to create awareness among the common peoples who are chewing pan regularly.

**Keyword:** Bacteria, Betel leaf, antibiotic, resistant, contamination, heterotrophic.

## 1. INTRODUCTION

An excellent mouth freshener, the deep green young heart shaped leaves of Betel (*Piper betel* L.) vines are popularly known as Pan in India. It is related with every human life. This edible leaves are traditionally used for chewing for mouth fresh in their natural raw condition. The *Piper betel* L. (Betel leaf) is the leaf of a vine belonging to the Piperaceae family i.e. the Black Pepper family (Gunther, 1952), which includes pepper and Kava. There are about 100 varieties of betel vine is available throughout the world, of which about 40 varieties are found in Indian territory and about 30 verities are reported from the boundary of West Bengal (Guha 1997, 2006; Maity 1989; Samanta 1994). The Paan chewing is much popular among the village people than that of urban areas.

It grows best under the shaded, tropical forest ecological conditions with a humidity and temperature ranging from 40-80% and 15-40°C, respectively. A well-drained fertile sandy or sandy loam or sandy clay soil with pH range of 5.6 –8.2 is considered suitable for its cultivation (CSIR, 1969; Guha and Jain, 1997). However, in the areas with lower rainfall (1500 – 1700 mm) the crop is cultivated with small and frequent irrigations, i.e. every day in summer and every 3-4 days in winter, whereas adequate drainage is required during the rainy season (Jana, 1995; Mishra *et al*., 1997).In different corner of the country it is also known as Nagaballi, Nagurvel, Saptaseera, Sompatra, Tamalapaku, Tambul, Tambuli, VakshaPatra, Vettilaietc (CSIR, 1969; Guha, 1997). The best Betel leaf is the "Magadhi" variety (from the Magadha region) grown near Patna in Bihar, India. In Kerala, the famous variety of betel leaf is from Venmony near Chengannur and it is called "Venmony Vettila". Betel leaf cultivated in Tirur,in Kerala, Hinjilicut in Odisha are of fine quality.
Betel leaves exported from Tirur are famous in Pakistan as "Tirur Pan".
The vast economic potentiality of the crop can be adequately established by the fact that about 15-20 million people consume betel leaves in India on a regular basis (Jana, 1996) besides those in other

countries of the world which may include over 2 billion consumers. Further, as far as the national employment generation is concerned, it is estimated that about 20 million people derive their livelihood directly or indirectly, partly or fully from production, processing, handling, transportation and marketing of betel leaves in India, which includes about 5 million workers from West Bengal (Jana, 1995; Jana, 1996).

The active constitution of betel oil is belongs to the primarily class ally benzene compounds Though particular emphasis has been placed on chavibetol (betel-phenol; 3-hydroxy-4-methoxyallylbenzene), it also contains chavicol (p-allyl-phenol; 4-allyl-phenol), estragole (p-allyl-anisole; 4-methoxy-allylbenzene), eugenol (allylguaiacol; 4-hydroxy-3-methoxy-allylbenzene; 2-methoxy-4-allyl-phenol), methyl eugenol (eugenol methyl ether; 3,4-dimethoxy-allylbenzene), and hydroxycatechol (2,4-dihydroxy-allylbenzene) (CSIR,1984). Except these there are several terpenes and terpenoids are also found in the betel oil. There are like two mono-terpenes, p-cymene and terpinene, and two mono-terpenoids, eucalyptol and carvacrol. Additionally, some other are two sesquiterpenes, cadinene and caryophyllene.

Fathilah *et al*. (2000) have reported that the crude aqueous extract of *Piper betle*L. Leaves exhibits antibacterial activity towards *Streptococcus mitis*, *Streptococcus sanguis* and *Actinomyces viscosus*, some of the early colonizers of dental plaque.

The most probable place of origin of betel vine is Malaysia (Chattopadhyay and Maity, 1967) and also grows in South and South –East Asia (India, Sri Lanka and Bangladesh).

The two key current health problem facing the under development countries are poor nutrition with in adequate water supply and sanitation. It is this problem that underlay the poor condition of the host and hence high level of infectious disease. It has estimated that as much as 80% of disease in under developed countries is associated with water. Few people are access to an adequate quantity or quality of water supply or to an effective sewage disposal. These applies to both crowded urban and the rural areas and the result is the high level of faecally related disease such as hookworm, cholera and chronic dysentery. The insanitary condition under which they live produces adverse effect on the health of the individuals. Generally speaking, the clerical staff, office bearers, porters, cart pullers and others are more liable to enteric diseases as they often take food and drinks outside as well as accustomed to chewing pan from road side pan stalls and thereby becomes promote to infection. Very often pain sellers do not follow the general guidelines of personal cleanliness, thus creating conditions favorable for the transmit tent and proliferation of water born diseases. This neglect to follow the general rule of personal hygiene is not entirely due to ignorance, but to the traditional habits and customs of the people. Due to scarcity of filtered water in summer, people have to use unfiltered water for cleaning purposes. The common practice of washing the pan with the contaminated water may also help in the spread of enteric diseases.

Due to the serious implication from the consumption of contaminated pan, this work aims to conducting road side pan stall survey on pan washed water in five major places in North Bengal to identify the associated antibiotic resistant bacteria and their resistant pattern to create awareness among the mass people and physician on indiscriminate use of antibiotics.

## 2. MATERIALS AND METHODS

**2.1 SURVEY AREA:** The surveys have been performed mainly from the Northern part of the West Bengal. Paan chewing is very much popular throughout the North Bengal as other part of India. To perform this work in better way five places of North Bengal were selected. The samples were collected from six locations. Those locations are, Islampur, Bagdogra, Sivmandir, Jalpaigury, and coochbehar. The maximum people of these areas are found to chewing pan. The peoples who are very much dependent on chewing *pan* are mainly belongs to poor villagers, vendors, labours and some common peoples. The *pan* stalls are very much frequent in these study areas mainly along with the different roads, Bus stand, Railway station, Cinema hall and Market places.

**2.2. COLLECTION OF THE SAMPLE:** Pre sterilized and paper wrapped bottles were used for sampling .While sampling in tumbler, the bottle (still stopped) is slinked in water (at a depth of 15 – 30 c.m.). The bottle was held thereby the base in one hand, while with the other hand, the stopper and cover was removed together. The stopper was retain in the hand while the bottle is filled (nearly3/4) and the stopper was replaced tightly and the filled bottle was taken out from the water .The bottle was not filled completely, as air- space of about 3 c.m. was allowed. The collected samples were then transported to the laboratory and the analyses were performed within few hours.

**2.3. MOST PROBABLE NUMBER TEST (MPN):** The most probable number (MPN) concept for estimating bacteria are used on multiple dilutions to extinction *i.e.* subdivision of the sample and is

valuable for estimating the population of microorganisms where other bacterial species predominate, *e.g.* in contaminated water. The MPN method is most frequently used for determining coli form counts but specific types of other organisms can also be enumerated.

**2.3.1. METHOD:**
10 ml of the LB 2X and LB 1X medium were added to the test tube .9ml was added first then, the remaining was added to the Durham tube which was then again inverted into the culture tubes. After that 10ml of sample was added to the LB 2x cultured tubes and 1ml and 0.1ml to will be 1x culture tubes (5+5+5 tubes).Then these tubes were incubated at $37^0$c for 48hrs.The gas form in the Durham tubes indicated the present of coliform bacteria

**2.4. TOTAL HETEROTROPHIC LOAD:** Total heterotrophic load of bacteria in collected water sample is enumerated by standard plate count (SPC)

**2.4.1. STANDARD PLATE COUNT (SPC):** The standard plate count values provides density of aerobic and facultative bacteria in water sample which can grow at $37^0$c.The SPC values are useful in warning about excessive microbial growth in any water sample and also in judging the efficiency of water and waste water treatment in removing microorganisms. The method involves inoculating water sample of different dilution (0, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$) on a suitable medium (LB) in the sterile Petridis and counting the colonies develop after incubation ($37^0$for 24hrs).

**2.5. QUANTIFICATION OF SELECTIVE MEDIA:** Collected samples were grown for quantitative test and plating on different selective and high medium i.e., ENDO-agar, EMB-agar, MAC –Conkey Media, Xylose Lysine agar, Leifsons Deoxycholate agar, Pfizer Selective Enterococcus agar and Bismuth Sulphate Agar followed by Tong & Sadowsky (1994), Endo (1904), Levin &Schoenlein (1930), Clesceri et al (1998), and Marshall (1992).

**2.5.1. ENDO – AGAR MEDIA:** The method was performed for this test that suggested by Apha (1998) and Endo (1904).
ENDO – Agar is recommended for the confirmation of the presumptive- test for members of the coliform group. Tubes of liquid media showing gram positive ('+'ve) presumptive reaction for coliform are sub-cultured on two ENDO -Agar lactose fermenting coliform give to red colonies with metallic seen while lactose non – fermenters form colourless colonies .

**2.5.2. EMB – AGAR MEDIA: The** method was performed for this test that suggested by Marshall (1993), Downes and Ito (2001), Baron et al (1959).
EMB-Agar is used for the isolation and differentiation of gram negative ('-'ve), *Enteric basili.* The ratio of eosin yellow & methyle blue is adjusted to give best differentiation between lactose fermenters and non–fomenters with minimum toxicity. Sucrose is included in this medium to detect their number of the coliform group which ferments this carbohydrate rapidly than lactose.

**2.5.3. MAC –CONKEY MEDIA:** The method was performed for this test that suggested by Luis (2004).
The MAC- Conkey media has been recommended use in microbiological examination of water sample for coliform counts. The selective action of this medium is attributed to crystal violet and bile salts which are inhibitory to most species of gram negative ('-'ve) bacteria. Gram negative bacteria usually grow in the medium and are differentiated by this ability to ferment lactose. Lactose fermenting strains grows as red or pink or may be surrounded by a zone of acid precipitate bile. The red colour is due to the production of acid from lactose, absorption neutral red and subsequent colour changes of the dye when the $P^H$ of the medium falls.

**2.5.4. XYLOSE LYSINE DEOXYCHOLATE AGAR (XLD):** The method was performed for this test that suggested by Apha (1992), Bhat*et al* (1975), Dunn *et al* (1971) and Rollender (1969).This mediais used for selective isolation and enumeration of *Salmonella typhi* and other *Salmonella* sp. in accordance with BP.

**2.5.5. LEIF SONSDEOXYCHOLATE AGAR (LDA):** The method was performed for this test that suggested by Kwon-Chung and Bennett (1992), Taplin*et al.* (1969) and Larone (1995). This media is used for selective isolation and differentiation of *Salmonella* species and *Shigella* species.

**2.5.6. PFIZER SELECTIVE ENTEROCOCCUS AGAR (PSEA):** This is also a high medium and it was suspended 58gm in 1000 ml of distilled water. Heat to dissolve the medium completely and sterilized by autoclave at 15 lb pressure for 15 minutes. It is used for selective isolation and cultivation of *Enterococci*.

**2.5.7. BISMUTH SULPHATE AGAR (BSA):** This is a high medium and it was suspended 52.33 gm in 1000 ml of distilled water .Water boiled to dissolved the medium completely .Do not autoclave or overheat. It cool to 50-550c and mixed well to

dispersed precipitated Bismuth Sulphate in suspension and pour thick plates. This is used for isolation and preliminary identification of *Salmonella typhi* and others *Salmonella* sp. in urine, sewage and food etc.

**2.6. DETERMINATION OF MULTIPLE ANTIBIOTIC RESISTANCE BACTERIA OF THE ISOLATES:** In this experiment the incidence of total heterotrophic bacterial load from a site was investigated. Total viable count of bacteria contain in water sample were also plated separately on Luria agar plate containing standard concentration of antibiotics. Resistance colonies were selected under the respective antibiotic plates and were randomly picked up to constructed Master plate for replica plating on 4 or 5 different antibiotic plates and scores different combination of antibiotic resistance pattern.

**2.6.1. PROCEDURE:** The multiple antibiotic resistant bacteria are counted in the following way.

**1st day:** Total 35 Luria agar plates were prepared which were supplemented with five types of antibiotics namely Amphicillin (Amp) =100 µg / ml Chloramphenicol (Chl) =50 µg / ml Kanamycin (Kan) =50 µg / ml Streptomycin (Strep) = 100 µg / ml Tetracyclin (Tet) =20 µg / ml

**2nd day:** The resistant colonies grows on each of the seven different selective plates (XLD, BSA, LDA, PSA, EMB, ENDO & MAC) were picked up with the help of the sterile tooth picks and were Transferred to 5 different gridded LA – plates to prepare the Master plates. The Master plates were then kept in the incubator at 37 ˚C.

**3rd day:** Replica of each Master plate was made onto the Luria agar plates containing antibiotics, as follows:

Master plates constructed with the colonies that were picked from original MAC plates and gridded on L.B. agar are used for replica plating on the L.A. medium containing different antibiotics and make total 35 different LB plates with 5 antibiotics. After passing these plates in the valvate – covered wooden-block, they acquire some bacteria on the same position as on the master plates. These plates are now incubated for overnight at 37°C.

**4$^{th}$ day:** The distinct bacterial colonies which appeared on the different antibiotic plates were tallied against the gridded master plate and in this way the multiple drug-resistance bacteria were scored.

**3. RESULT & DISCUSSION**

During this study, collected *paan* wash water samples from the tumbler where paan is being washed before it is prepared and given to the customers. The collected samples were analyzed to investigate the incidence of fecal coliform and fecal Enterococci. Besides calculating the MPN value, recoded total heterotrophic bacterial load, the presence of specific selective micro organism in different selective plates. The present study also recoded the antibiotic resistance profile to those isolates.

**3.1. MPN TEST FOR THE POPULATION OF MICRO-ORGANISM FROM COLLECTED WASH-WATER SAMPLE:**

In this study, a total of five samples have been collected from Shibmandir, Bagdogra, Jalpaigury, Islampur and Siliguri. These collected samples were analyzed in laboratory and shows the following results of MPN test.

**Sample- 1:** For the MPN test a total of 15 tubes for Sample- 1 have been taken in three different dilutions (10ml, 1 ml and 0.1 ml) for TC, FC and FS. Result shows for positive combination is 5-5-5 for TC and FC but in case of FS the positive combination is 5-4-2. The MPN index for TC and FC against 5-5-5 combination is ≥1600 and 95% confidence limit in lower and upper shows nil. But in case of FE MPN index is 220 and 95% confidence limit in lower and upper shows 100, 580 respectively.

**Sample-2:** For the MPN test a total of 15 tubes forSample-2havebeen taken in three different dilutions (10ml, 1mland0.1ml) for TC, FC and FE. Result shows for positive combination is 5-5-5. The MPN index against 5-5-5 combination is ≥1600 and 95% confidence limit in lower and upper shows nil.

**Sample- 3:** For the MPN test a total of 15 tubes for Sample- 3 have been taken in three different dilutions (10ml, 1 ml and 0.1 ml) for TC, FC and FE. Result shows for positive combination is 5-5-5. The MPN index against 5-5-5 combination is ≥1600 and 95% confidence limit in lower and upper shows nil.

**Sample- 4:** For the MPN test a total of 15 tubes for Sample-4 have been taken in three different dilutions (10ml, 1 ml and 0.1 ml) for TC, FC and FE. Result shows for positive combination is 5-5-5 for TC and FC but in case of FE the positive combination is 4-4-0. The MPN index for TC and FC against 5-5-5 combination is ≥1600 and 95% confidence limit in lower and upper shows nil. But in case of FE MPN index is 34 and 95% confidence limit in lower and upper shows 16, 80 respectively.

**Sample- 5:** For the MPN test a total of 15 tubes for Sample-5 have been taken in three different dilutions (10ml, 1 ml and 0.1 ml) for TC, FC and FS. Result shows for positive combination is 5-5-5 for TC and FC but in case of FE the positive combination is 5-4-2. The MPN index for TC and FC against 5-5-5 combination is ≥1600 and 95% confidence limit in lower and upper shows nil. But in case of FS MPN

index is 220 and 95% confidence limit in lower and upper shows 100, 580 respectively. MPN results of all samples are shown in table no -1.

**3.2. TOTAL HETEROTROPHIC LOAD FROM COLLECTED WASH-WARE SAMPLE:** Through standard plate count values the heterotrophic load i.e., density of aerobic and facultative bacteria in different wash water sample have been determined at $37^{0c}$. Five water sample of different dilution such as 0, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ have been taken and inoculating on a suitable medium (LB) in the sterile Petridis and counting the colonies develop after incubation. After 24hrs of incubation at $37^0$C, SPC values were calculated. It is found that the $10^{0}$ dilution shows the maximum load and it is gradually reduced against serial dilution $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4.}$ Sample wise study for total c.f.u/ml shows maximum load i.e. $27.8 \times 10^{5}$ in Sample 4, whereas$23.2 \times 10^{5,}12.1 \times 10^{5}$ , $6.64 \times 10^{5}$,$5.85 \times 10^{5}$ in Sample 6, 5, 3,2 respectively. Results of total heterotrophic load shown in table no-2.

**3.3. QUANTIFICATION OF SELECTIVE MEDIA TO IDENTIFY THE PARTICULAR BACTERIA GROUP:** The different plating media are used to identify of a particular group of bacteria. To determine the existing bacterial group in different wash water sample, the media like Endo-agar, Mac-Conkey agar, EMB-agar and four High media i.e, BSA, XLD, LDA and PSE-agar were used. The media wise results were discussed below.

**ENDO – AGAR MEDIA:** ENDO-Agar is recommended for the confirmation of the presumptive- test for members of the
Coliform group.Here *E.aerogens* grow Red and *E. coli* grow Red with metallic seen Colour colony
On the ENDO – Agar Media.

**EMB – AGAR MEDIA:** EMB agar is used for the isolation and differentiation for Gram negative *Enteric bacilli*.Here *E. aerogenes*grow Pink, no seen purple with black center and *K. pneumonia* growDark center and greenic metalliccolour on the media.

**MAC-CONKEY MEDIA:**
This method has been recommended for the use of microbiological examination of water sample for coliform counts of gram negative coliform bacilli. Here *E.coli* grow Pink to red colour, *E.aerogenes* grow Pink to meceoid colour, *S. faecalis, and S.aureus* grow pink to red colour on the media.

**XYLOSE LYSINE DEOXYCHOLATE AGAR (XLD):** This is a high media and it was suspended 55gm in 1000ml water. Here the dissolve the medium completely cool and pours into plates. This is used for selective isolation and enumeration of *Salmonella typhi* and other *Salmonella* species in accordance with BP.

**LEIF SONS DEOXYCHOLATE AGAR (LDA):**
This is a high media and it suspended 48.5 gm in distilled water. Boil to dissolve the medium completely. Do not autoclave or over heat the medium. This is used for selective isolation and differentiation of *Salmonella* species and *Shigella* species.

**PFIZER SELECTIVE ENTEROCOCCUS AGAR (PSEA):** This is also a high medium and it suspended 58gm in 1000 ml distilled water. Heat to dissolve the medium completely and sterilized by autoclave at 15 lb pressure for 15 minutes. It is used for selective isolation and cultivation of *Enterococci*.

**BISMUTH SULPHATE AGAR (BSA):** This is a high medium and it suspended 52.33 gm in 1000 ml distilled water .Water boiled to dissolved the medium completely .Do not autoclave or overheat. It cool to 50-550c and mixed well to dispersed precipitated Bismuth Sulphate in suspension and pour thick plates. This is used for isolation and preliminary identification of *Salmonella typhi*, and others *Salmonella* species in urine, sewage and food etc. Results of particular bacteria in different media shown in table no-3.

**3.3.1. STANDARD PLATE COUNT ON DIFFERENT SELECTIVE MEDIA**
Standard plate count values on different selective media were recorded for each sample. To perform this experiment, three different dilution ($10^{0}$, $10^{-1}$ & $10^{-2}$) of each 5 sample have been prepared and grow in 7 media (three selective and 4 high media) simultaneously.

**Sample 1:** In Sample 2, the result shows that the EMB media with maximum load i.e. $968 \times 10^{2}$ c.f.u/ml where as other media with value shows $105 \times 10^{2,}112 \times 10^{2}$,$70 \times 10^{2}43 \times 10$, $380 \times 10^{2}$ and $516 \times 10^{2}$againstXLD,BSA, LDA,PSE,ENDO and MAC respectively.

**Sample 2:** In Sample 3, the result shows that the MAC media with maximum load i.e. $1340 \times 10^{3}$c.f.u/ml where as other media with value shows$314 \times 10^{2}$ , $102 \times 10^{3}$ , $27 \times 10^{3}$ , $22 \times 10^{2}$and $405 \times 10^{3}$against,XLD,BSA, LDA, PSE, and ENDO media respectively.

**Sample 3:** In Sample 4, the result shows that the BSA media with maximum load i.e.$1123\times 10^{2}$ c.f.u/ml where as other media with value shows$412 \times 10^{2}$, $362 \times 10^{2}$ ,$43 \times 10^{2}$ ,$569 \times 10^{3}$ , and $720 \times 10^{2}$against,XLD, LDA,PSE,ENDO and EMB media respectively.

**Sample-4:** In Sample 5, the result shows that the MAC media with maximum load i.e131 × 10 $^{3}$c.f.u/ml where as other media with value shows 33 × 10 $^{2}$,218 ×10,202 × 10,and 73 × 10,against, XLD, LDA, PSE, ENDO and EMB media respectively.

**Sample 5:** In Sample 6, the result shows that the MAC media with maximum load i.e.105 × 10 $^{3}$c.f.u/ml where as other media with value shows 184 × 10 $^{2}$,43 × 10 $^{2}$,269 × 10 $^{2}$,and 319 × 10against,XLD, LDA,PSE,ENDO and EMB media respectively. Results of Standard plate count on different selective media for five samples shown in table no -4.

**3.4. DETERMINATION OF MULTIPLE ANTIBIOTIC RESISTANCE BACTERIA OF THE ISOLATES:** Antibiotics have been developed to treat disease caused by microorganism in humans, animals; however, these antibacterial agents are losing their effectiveness because of their spread and persistence of drug resistant organisms. The present situation, we may find a time when such agents are no longer useful to combat disease. It is due in large part to the indiscriminate use of antibiotics.

**3.5 RESULT OF REPLICA PLATING:**

**Sample -1**

**BSA –AGAR:** Master plate BSA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 75 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 26 isolates shows maximum resistance in Triple combination (34.66%) followed by 3 isolates shows Sensitive to all antibiotics, 5 isolates response on Quadruple, 19 isolates response on Single combination, 4 isolates response on Quintuple and 18 response on Double combination.

**XLD –AGAR:** Master plate XLD - agars constructed colonies were replica plated on to different antibiotic plates. Total of 73 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 38 isolates shows maximum resistance in Quadruple combination (52.05%) followed by 1 isolates shows Sensitive to all antibiotics, 24 isolates response on Triple, no isolates response on Single combination, 6 isolates response on Quintuple and 4 response on Double combination.

**LDA –AGAR:** Master plate LDA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 83 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 68 isolates shows maximum resistance in Triple combination (81.92%) followed by no isolates shows Sensitive to all antibiotics, 11 isolates response on Quadruple, 1 isolates response on Single combination, 1 isolates response on Quintuple and 2 response on Double combination.

**MAC–AGAR:** Master plate MAC - agars constructed colonies were replica plated on to different antibiotic plates. Total of 77 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 40 isolates shows maximum resistance in Triple combination (51.94%) followed by 6 isolates shows Sensitive to all antibiotics, 23 isolates response on Quadruple, 2 isolates response on Single combination, 1 isolates response on Quintuple and 5 response on Double combination. Summaries results of sample 1 shown in figure-1.

**Sample 2:**

**MAC–AGAR:** Master plate MAC - agars constructed colonies were replica plated on to different antibiotic plates. Total of 71 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quartile (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 51 isolates shows maximum resistance in Triple combination (71.83%) followed by 10 isolates shows Sensitive to all antibiotics, no isolates response on Quadruple, 2 isolates response on Single combination, 0 isolates response on Quintuple and 8 response on Double combination.

**BSA – AGAR:** Master plate BSA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 74 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quartile (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 48 isolates shows maximum resistance in Quadruple

combination (64.8%) followed by 3 isolates shows Sensitive to all antibiotics, 15 isolates response on Triple, 6 isolates response on Single combination, no isolates response on Quintuple and 2 response on Double combination.

**XLD –AGAR:** Master plate XLD - agars constructed colonies were replica plated on to different antibiotic plates. Total of 74 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quartile (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 44 isolates shows maximum resistance in Double combination (59.45%) Followed by 3 isolates shows Sensitive to all antibiotics, no isolates response on Quadruple &Quintuple, 5 isolates response on Single combination, 22 isolates response on Triple combination.

**XLD–AGAR:** Master plate XLD – agars constructed colonies were replica plated on to different antibiotic plates. Total of 76 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 55 isolates shows maximum resistance in Triple combination (72.3%) followed by no isolates shows Sensitive to all antibiotics, 8 isolates response on Quadruple, 0 isolates response on Quintuple, 6 isolates response on Single combination, and 7 response on Double combination. Summaries results of sample 2 shown in figure-2.

**Sample-3:**

**MAC–AGAR:** Master plate MAC - agars constructed colonies were replica plated on to different antibiotic plates. Total of 77 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 52 isolates shows maximum resistance in Triple combination (67.4%) followed by 2 isolates shows Sensitive to all antibiotics, no isolates response on Quadruple, 9 isolates response on Single combination, 1 isolates response on Quintuple and 13 response on Double combination.

**BSA - AGAR:**Masterplate BSA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 76 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 30 isolates shows maximum resistance in Triple combination (39.4%) followed by 19 isolates shows Sensitive to all antibiotics, 3 isolates response on Quadruple, 11 isolates response on Single combination, 0 isolates response on Quintuple and 13 response on Double combination.

**LDA –AGAR:** Master plate LDA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 75 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 30 isolates shows maximum resistance in Triple combination (39.9%) followed by 0 isolates shows Sensitive to all antibiotics, 4 isolates response on Quadruple, 23 isolates response on Single combination, 2 isolates response on Quintuple and 16 response on Double combination.

**XLD–AGAR:** Master plate XLD – agars constructed colonies were replica plated on to different antibiotic plates. Total of 77 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 40 isolates shows maximum resistance in Triple combination (51.89%) followed by 3 isolates shows Sensitive to all antibiotics, no isolates response on Quadruple, 10 isolates response on Single combination, 0 isolates response on Quintuple and 24 response on Double combination. Summaries results of sample 3 shown in figure-3.

**Sample-4:**

**MAC–AGAR:** Master plate MAC - agars constructed colonies were replica plated on to different antibiotic plates. Total of 32 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 14 isolates shows maximum resistance in Triple combination (43.29%) followed by 0 isolates shows Sensitive to all antibiotics, 5 isolates response on Quadruple, 3 isolates response on Single combination, 1 isolates response on Quintuple and 9 response on Double combination.

**BSA – AGAR:** Master plate BSA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 39 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 14 isolates shows maximum resistance in Triple combination (41.17%) followed by 0 isolates shows Sensitive to all antibiotics, no isolates response on Quadruple, 9 isolates response on Single combination, 0 isolates response on Quintuple and 11 response on Double combination.

**LDA –AGAR:** Master plate LDA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 20 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 8 isolates shows maximum resistance in Double combination (40%) followed by 0 isolates shows Sensitive to all antibiotics, no isolates response on Quadruple, 6 isolates response on Single combination, 4 isolates response on Quintuple and 2 response on Triple combination.

**XLD –AGAR:** Master plate XLD - agars constructed colonies were replica plated on to different antibiotic plates. Total of 67 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 55 isolates shows maximum resistance in Single combination (82%) followed by 0 isolates shows Sensitive to all antibiotics, no isolates response on Quadruple, 0 isolates response on Triple combination, 0 isolates response on Quintuple and 12 response on Double combination. Summaries results of sample 4 shown in figure-4.

**Sample-5:**

**MAC–AGAR:** Master plate MAC- agars constructed colonies were replica plated on to different antibiotic plates. Total of 30 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 14 isolates shows maximum resistance in Triple combination (46.6%) followed by 0 isolates shows Sensitive to all antibiotics, 4 isolates response on Quadruple, 0 isolates response on Single combination, 0 isolates response on Quintuple and 12 response on Double combination.

**BSA –AGAR:** Master plate BSA - agars constructed colonies were replica plated on to different antibiotic plates. Total of 61 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 59 isolates shows maximum resistance in Triple combination (96.7%) followed by 0 isolates shows Sensitive to all antibiotics, 1 isolates response on Quadruple, 0 isolates response on Single combination, 0 isolates response on Quintuple and 1 response on Double combination.

**LDA – AGAR:** Master plate LDA–agars constructed colonies were replica plated on to different antibiotic plates. Total of 20 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 8 isolates shows maximum resistance in Double combination (40%) followed by 0 isolates shows Sensitive to all antibiotics, 1 isolates response on Quadruple, 7 isolates response on Triple combination, 0 isolates response on Quintuple and 4 response on Single combination.

**XLD –AGAR:** Master plate XLD - agars constructed colonies were replica plated on to different antibiotic plates. Total of 50 isolates were plated on different antibiotics and counted the colonies. After the incubation period combination patter of resistance shows that Quintuple (ACKST), Quadruple (ACST, ACKT), Triple (ACT, AST), Double (AT, AC. AS, CT), Single (A & S) and Sensitive to all antibiotics. 17 isolates shows maximum resistance in Quadruple combination (56%) followed by 1 isolates shows Sensitive to all antibiotics, 4 isolates response on Single, 0 isolates response on Triple combination, 0 isolates response on Quintuple and 0 response on Double combination. Summaries results of sample 5 shown in figure -5.

## CONCLUSION

From this study it is concluded that the water used by paan vendors of road side paan stall is contaminated with many antibiotic resistant pathogenic bacteria like *Salmonella typhi, S. faecalis, S.aureusand E. aerogenes, Shigella sp., K. pneumoniae, E.coli, P. mirabilis* and *Enterococci* from different paan wash water sample. Specific media isolates were applied to

identify the pathogenic bacteria from the collected wash water paan sample.

The present study also investigates that how those pathogenic bacteria are transmitted from paan wash water from road side paan vendor to human body and causing various diseases. When paan vendors supply their customer order to serve paan for chewing those antibiotic resistant pathogenic bacteria are transmitted into human body through readymade paan. Most of the cases it is observed that water of the tumbler which is used for wash of paan in paan stall bears so many pathogenic organism. As the tumblers are open to air, air connected bacteria easily contaminated with water which is used for long time to wash betel leaf. Sometimes water source is also reliable for the presence of those bacteria and other pathogenic organism in paan wash water as it is collected from different sources.

**ACKNOWLEDGEMENT:** The author is grateful to Dr. Ranadhir Chakraborty, Microbiology Laboratory of Botany Department, and University of North Bengal for his constant advice and suggestion of this study.

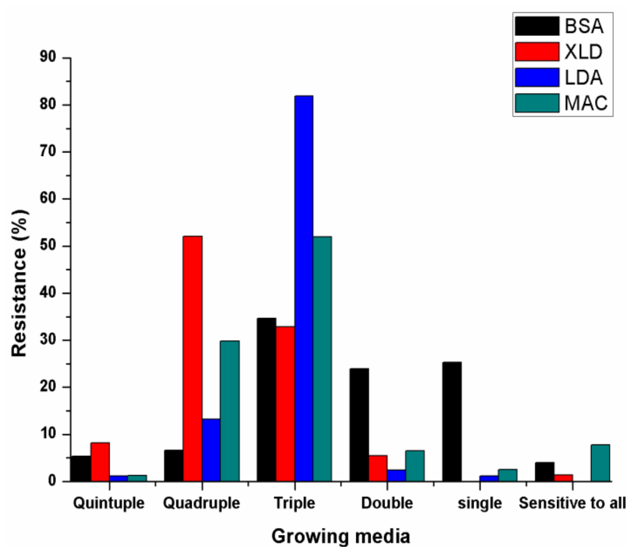


**Figure-1: Graphical representation of resistance with antibiotic combinations at different growth media.**

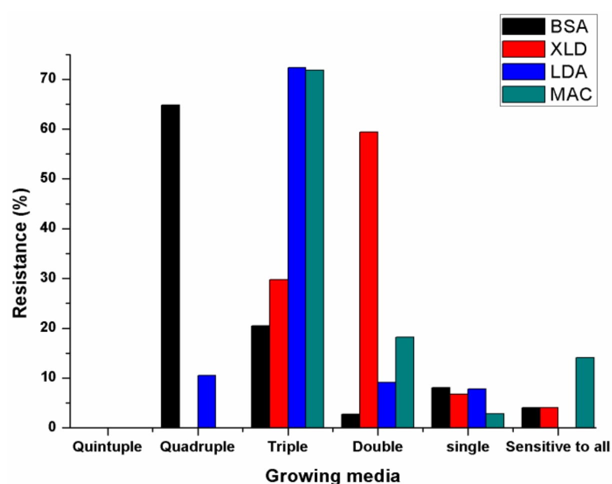


**Figure-2: Graphical representation of resistance with antibiotic combinations at different growth media.**

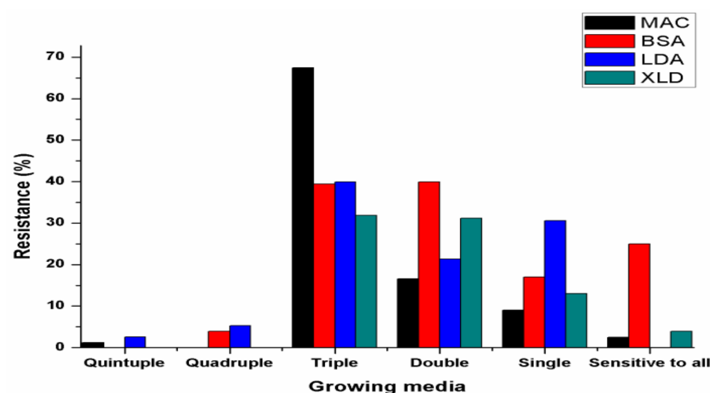


**Figure-3: Graphical representation of resistance with antibiotic combinations at different growth media.**

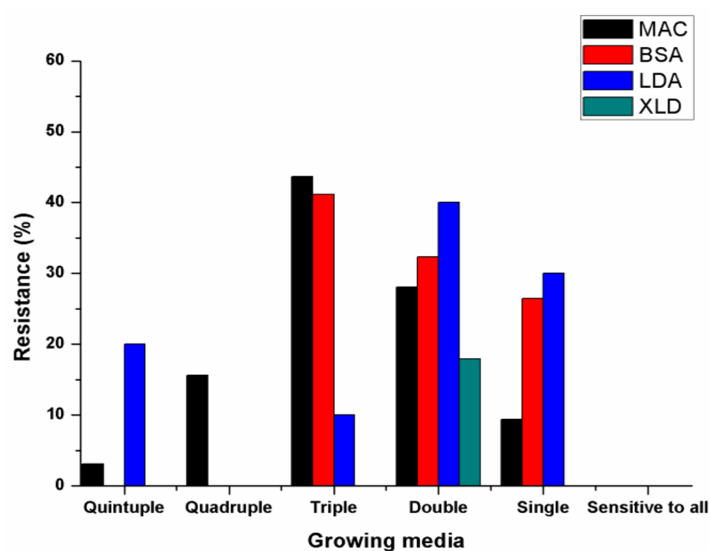


**Figure-4: Graphical representation of resistance with antibiotic combinations at different growth media.**

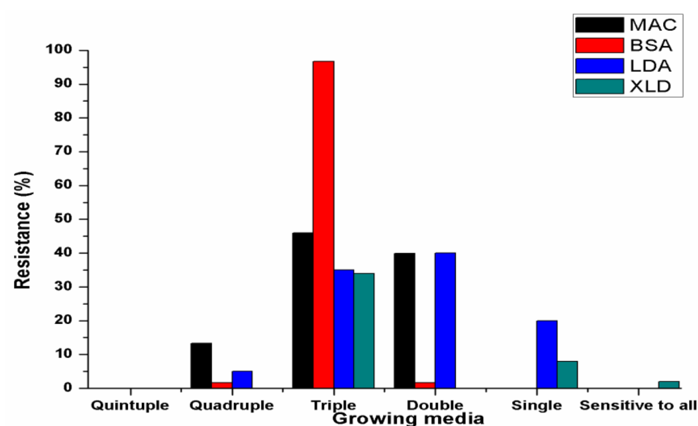


**Figure-5: Graphical representation of resistance with antibiotic combinations at different growth media.**

**Table 1: Results of MPN test for five Samples.**

| | Name of test | No. of tubes giving positive results | | | MPN | 95% confidence | |
|---|---|---|---|---|---|---|---|
| | | 5 of 10 ml tube | 5 of 1 ml tube | 5 of 0.1 ml tube | | Lower | Upper |
| Sample 1 | T.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.E | 5 | 4 | 2 | 220 | 100 | 580 |
| Sample 2 | T.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.E | 5 | 5 | 5 | ≥1600 | - | - |
| Sample 3 | T.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.E | 5 | 5 | 5 | ≥1600 | - | - |
| Sample 4 | T.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.E | 4 | 4 | 0 | 34 | 16 | 80 |
| Sample 5 | T.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.C | 5 | 5 | 5 | ≥1600 | - | - |
| | F.E | 5 | 4 | 2 | 220 | 100 | 580 |

**Table 2: Standard plate count for total Heterotrophic load**

| Sample No. | Dilution series | | | | | Total c.f.u / ml. |
|---|---|---|---|---|---|---|
| | $10^{0}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | |
| 1 | $585 \times 10^{2}$ | $585 \times 10$ | 585 | 40 | | $5.85 \times 10^{5}$ |
| 2 | $664 \times 10^{2}$ | $664 \times 10$ | 664 | 111 | 28 | $6.64 \times 10^{5}$ |
| 3 | $278 \times 10^{3}$ | $278 \times 10^{2}$ | $278 \times 10$ | 278 | 20 | $27.8 \times 10^{5}$ |
| 4 | $121 \times 10^{3}$ | $121 \times 10^{2}$ | $121 \times 10$ | 121 | 13 | $12.1 \times 10^{5}$ |
| 5 | $232 \times 10^{3}$ | $232 \times 10^{2}$ | $232 \times 10$ | 232 | 22 | $23.2 \times 10^{5}$ |

**Table 3: Growth of bacteria against different media.**

| **Media** | ***E.coli*** | ***E.aerogens*** | ***K.pneumoniae*** | ***S.faecalis*** | ***S.aureus*** | ***S.typhi*** | ***Shigellasp*** |
|---|---|---|---|---|---|---|---|
| ENDO-Agar | + | + | - | - | - | - | - |
| EMB- Agar | + | + | + | - | - | - | - |
| MAC-CONKEYAgar | + | + | + | + | + | - | - |
| XLD Agar | - | - | - | - | - | + | - |
| LDA Agar | - | - | - | - | - | + | + |
| PSE- Agar | - | + | - | - | - | - | - |
| BSA | - | - | - | - | - | + | - |
| | | | | | | | |

Here the full form of the experimented strains are *Escherichia coli, Enterobactor aerogens, Klebsialla pneumonia, Streptococcus faecalis, Staphylococcus aureus, Salmonella typhi, Shigella sp.*

**Table4: Standard plate count on different selective media for five samples.**

| Sample no. | XLD(Total c.f.u/ml) | BSA(Total c.f.u/ml) | LDA(Total c.f.u/ml) | PSE(Total c.f.u/ml) | ENDO(Total c.f.u/ml) | MAC(Total c.f.u/ml) | EMB(Total c.f.u/ml) |
|---|---|---|---|---|---|---|---|
| 1 | $105 \times 10^{2}$ | $112 \times 10^{2}$ | $70 \times 10^{2}$ | $43 \times 10$ | $380 \times 10^{2}$ | $516 \times 10^{2}$ | $968 \times 10^{2}$ |
| 2 | $314 \times 10^{2}$ | $102 \times 10^{3}$ | $27 \times 10^{3}$ | $22 \times 10^{2}$ | $405 \times 10^{3}$ | $1340 \times 10^{3}$ | $964 \times 10^{2}$ |
| 3 | $412 \times 10^{2}$ | $1123 \times 10^{2}$ | $362 \times 10^{2}$ | $43 \times 10^{2}$ | $350 \times 10^{2}$ | $569 \times 10^{3}$ | $720 \times 10^{2}$ |
| 4 | $33 \times 10^{2}$ | $218 \times 10$ | $202 \times 10$ | $73 \times 10$ | $305 \times 10^{2}$ | $131 \times 10^{3}$ | $850 \times 10^{2}$ |
| 5 | $184 \times 10^{2}$ | $43 \times 10^{2}$ | $269 \times 10^{2}$ | $319 \times 10$ | $375 \times 10^{2}$ | $105 \times 10^{3}$ | $710 \times 10^{2}$ |

## REFERENCES


1. Gunther E. The Essential Oil, New York ; D Van Nostrand & Co. Inc., 1952; 5:160-161.
2. Guha P. Paan Theke Kutir Silpa Sambhabana, (In Bengali). Exploring Betel Leaves for Cottage Industry, In: Krishi Khadya-O- Gramin Bikash Mela –A Booklet published by the Agricultural and Food Engineering Department, IIT, Kharagpur, India, 1997; 15-19.
3. Guha P. Betel leaf: the neglected green gold of India, Agricultural and Food Engineering Department, IIT, Kharagpur, India. J. Hum. Ecol, 2006; 19 (2): 87-93.
4. Maity S. Extension Bulletin: The Betelvine, All India Coordinated Research Project on Betel vine, Indian Institute of Horticultural Research, Hessar ghatta, Bangalore, India, 1989.

5. Samanta C, Paan chaser samasya bali-o-samadhan: Ekti samikkha (In Bengali), A Report on the Problems and Solutions of Betel Vine Cultivation. A booklet published by Mr. H. R. Adhikari, C-2/16, Karunamoyee, Salt Lake City, Kolkata-64 (WB), India, 1994.
6. INDIA. (CSIR), The Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products - Raw Materials Vol viii: ph-re. India: Council for Scientific and Industrial Research (CSIR), 1969; 394.
7. Guha P, Jain RK. Status Report on Production, Processing and Marketing of Betel Leaf (Piper betle L.), Agricultural and Food Engineering Department, IIT, Kharagpur, India, 1997.
8. Jana BL. Gram Banglar Arthakari Phasal-Paan (In Bengali). Betel Leaf: A Cash Crop of Villages of Bengal. Asaboni, Flat 203, 184, B. B. Chatterji Road, Calcutta, 1995.
9. Jana B.L. Improved technology for betel leaf cultivation. A paper presented in the "Seminar-cum-Workshop on Betel leaf Marketing", held at State cashew nut farm, Directorate of Agricultural Marketing, Digha, Midnapur (W. B.), India, June 5-6,1996.
10. CSIR. The wealth of India, 1984; 8:87.
11. Mishra D, Mishra SK, Acharya A, Das JN. Annual Report of All India Coordinated Research Project on Betelvine. Department of Plant Pathology, College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar, India,1997.
12. Fathilah AR, Bakri MM, Rahim ZHA. Pascasidang Simposium Sains Kesihatan Kebangsaan ke-3. Fakulti Sains Kesihatan Bersekutu, UKM; 2000: 216-219.
13. Chattopadhyay SB, Maity S. Diseases of Betel vine and Spices. ICAR, New Delhi, 1967.
14. Tong Z, Sadowsky MJ. A selective medium for the isolation and quantification of Bradyrhizobium japonicum and Bradyrhizobium elkanii strains from soils and inoculants, Appl. Environ. Microbiol, 1994; 60:581.
15. Endo, Zentralbl, Bakteriol .Abt. 1, Orig, 1904; 35:109.
16. Levin, Schoenlein. A compilation of culture media for the cultivation of microorganisms, Williams & Wilkins, Baltimore, Md; 1930.
17. Clesceri, Greenberg, Eaton ed. Standard methods for the examination of water and wastewater, 20th ed. Washington, D.C, American Public Health Association; 1998.
18. Marshall Ed. Standard methods for the examination of dairy products, 16th ed. Washington, D.C, American Public Health Association; 1992.
19. [19] APHA, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, American Water Works Association and Water Pollution Control Federation, 20th ed., Washington; 1998.
20. Marshall Ed. Standard methods for the examination of dairy products, 16th ed. Washington, D.C, American Public Health Association; 1993.
21. Downes, Ito ED. Compendium of methods for the microbiological examination of foods, 4th ed. Washington, D.C, American Public Health Association; 2001.
22. Baron, Spilman, Carey. Bacteriol. Proc. 59$^{th}$ Gen. Meet. Soc. Am. Bacteriologists, Abstr. G7; 1959: 29.
23. Luis M, De LA,Maza, Pezzlo, Marie T, Janet T, Shigei, Peterson, Ellena M. Color Atlas of Medical Bacteriology, Washington, D.C, ASM Press;2004 :103.
24. APHA, American Public Health Association, Compendium of Methods for the microbiological Examination of Foods. - 3rd Ed; 1992.
25. Bhat P, Raina D. Comparative evaluation of deoxycholate citrate medium and xylose lysine deoxycholate medium in the isolation of *shigellae*. Am. J. Clin. Pathol, 1975; 64:99.
26. Dunn C, Martin, W. J. Comparison of media for isolation of Salmonellae and Shigellae from fecal specimens, European Pharmacopeia II, Chapter VIII, 10. Appl. Microbiol, 1971; 22:17.
27. Rollender W, Beckford O, Belsky R D, Kostroff B. Comparison of xylose lysine deoxycholate agar and Mac-Conkey Agar for the isolation of Salmonella and Shigella from clininal specimens, Am. J. Clin. Pathol, 1969; 51:284.
28. Kwon-Chung, Bennett, Lea, &Febiger. Philadelphia, Pa, Medical mycology: 1992.
29. Taplin, Zaias, Rebell, Blank, Arch. Dermatol, 1969;99:203.
30. Larone, Medically important fungi. A guide to identification, 3rd ed., Washington, D.C, American Society for Microbiology, 1995.