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ARTICLES

Research Articles

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Antibiogram of food-borne pathogens isolated from ready-to-eat foods and Zobo Drinks Sold Within and Around PRESCO Campus of Ebonyi State University (EBSU), Abakaliki, Ebonyi State, Nigeria

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Food poisoning (food-borne disease) is an infection that occurs after consuming food contaminated by sufficient numbers of viable pathogens and their toxins. It is a common and costly preventable infection that is of public health concern, and which is treated with available antibiotics. Jellof-rice, abacha, moi-moi and zobo drinks are some ready-to-eat foods sold within the PRESCO campus of Ebonyi State University (EBSU), Abakaliki, Nigeria. These foods are commonly patronized by students and other unsuspecting visitors in this region, and they have been implicated in a handful of bacterial related infections in recent times. Random samples of the food items were collected from shops selling them, and these were analyzed microbiologically to determine the most prevalent organisms. Suspect isolates were identified and tested for antibiotic susceptibility profiles. *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were the commonest microbes isolated, and these showed varying rates of resistance and susceptibility to the tested drugs. Clindamycin, ampicillin and ofloxacin were less effective against the test organisms while gentamicin, erythromycin and ciprofloxacin showed substantial activity. The findings in this study showed that some ready-to-eat foods and zobo drinks sold within PRESCO campus of EBSU, Abakaliki, Nigeria were considerably contaminated with resistant pathogenic bacteria, hence, the need for constant monitoring of ready-to-eat foods in order to prevent the outbreak of food-borne illnesses in this region.

Key words: Zobo drinks, ready-to-eat foods, bacteria, antibiotic resistance.

INTRODUCTION

According to the New South Wales Food Authority (NSWFA), ready-to-eat foods are foods that are originally consumed in the same state as that in which it is sold and does not include nuts in the shell and whole, raw fruits and vegetables that are intended for hulling, peeling or these foods are usually hazardous in that they support the growth of pathogens when not properly handled, prepared or stored; and they can serve as route for the onward transmission of food-borne pathogens in human population. Therefore, in as much as food supports life, it washing by the consumer (NSWFA, 2009). Some of has been described as a vehicle for the transmission of
microbial diseases, and among which are those caused by E. coli and other medically important bacteria (Ifediora et al., 2006; Kornacki et al., 2004; Muinde et al., 2005). According to the US Department of Health and Human Services (USDHHS) website, food-borne illnesses (which are commonly referred to as food poisoning) are diseases that results from eating contaminated food (USDHHS, 2013). Food poisoning can ensue after eating food contaminated by considerable number of viable pathogens, and this commonly occurs after eating at picnics, restaurants or fast food joint. Poor handling of these foods play critical role in the onward transmission of food-borne pathogens including Escherichia coli and Klebsiella pneumoniae to unsuspecting patrons who eat them. Bacterial pathogens have been implicated in a handful of food-borne diseases in recent times, and these microbes are resistant to some available antimicrobial agents (Kornacki et al., 2001; Ifediora et al., 2006; Elkholy et al., 2003). In addition, infections can also occur from toxin production by the organisms. Zobo drink is sourced from the water extract of dried calyx of Hibiscus sabdariffa plant (Haji-Faraji et al., 1999). It is an indigenous drink consumed in Africa, Asia and some parts of South America owing to its perceived medicinal benefits which include antioxidant effect, anti-diabetic effect, and anti-hypertensive effects (Kolawole et al., 2004; Lin et al., 2011; Fullerton et al., 2011). Some of the organisms that contaminate these foods are regarded as indicator organisms (for example, E. coli) due to their fecal origin (Kornacki et al., 2001). The growing resistance of pathogens (including E. coli and Klebsiella species) isolated from locally prepared ready-to-eat foods is a public health concern in both developed and developing countries (Elkholy et al., 2003). Bacterial-related resistant infections have posed a serious problem in the treatment of infectious diseases in health care delivery systems due to limited therapeutic options.

In view of this, this work is aimed at detecting the presence of some enteric pathogens from some ready-to-eat foods and zobo drinks sold within the PRESCO campus of Ebonyi State University, Abakaliki, Nigeria.

MATERIALS AND METHODS

Sample collection

Different types of ready to eat foods (how many samples collected) including rice (n = 50), abacha (n = 50), moi-moi (n = 50) and zobo drinks (n = 50) were aseptically and randomly collected from 20 food vendors within and around the PRESCO campus of Ebonyi State University (EBSU), Abakaliki, Nigeria. The samples were transported to the Microbiology Laboratory of EBSU, Abakaliki in transport media where they were analyzed following standard microbiology techniques.

Analysis of samples

Each food sample was macerated using a sterile marble mortar. One gram (1 g) of each food sample was homogenized in sterile water and the volume of the homogenate was made up to 10 ml to obtain a 1:10 suspension. 0.1 ml of the suspension was inoculated on Trypton Soy broth and incubated at 37°C for 18 to 24 h. A loopful of the culture was then transferred to MacConkey agar plates and incubated for 18 to 48 h at 37°C. Suspect colonies of E. coli and Klebsiella species were transferred to eosin-methylene blue (EMB) agar for proper differentiation. All growth media were procured from Oxoid (Oxoid, UK). Also, 10 fold serial dilutions of zobo drink samples were performed using each sample of zobo drink, and these were inoculated into nutrient broth. They were incubated for 18 to 24 h at 37°C. Loopful of the culture were transferred to MacConkey agar plates and EMB agar plates and were also incubated for 18 to 24 h at 37°C. Suspected colonies of E. coli and Klebsiella species were transferred to nutrient agar slants from which they were subjected to Gram staining, Indole test, Methyl red test, Voges proskauer and Citrate tests for proper identification (Cheesbrough, 2000).

Antibiogram

Antimicrobial susceptibility test was performed on Mueller-Hinton (MH) agar (Oxoid, UK) plates by the Kirby-Bauer disk diffusion method as per the Clinical Laboratory Standard Institute (CLSI) criteria (CLSI, 2010). The tested antibiotics included erythromycin (15 μg), ciprofloxacin (5 μg), ofloxacin (10 μg), gentamicin (10 μg), clindamycin (10 μg) and ampicillin (10 μg). All antibiotic disks were procured from Oxoid, UK. Standard strains of E. coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, K. pneumoniae ATCC 700603 were used as controls. Plates were incubated at 37°C, and zones of inhibition were measured using meter rule as per the CLSI criteria.

RESULT

E. coli were the most prevalent organism isolated from the ready-to-eat foods sold around the PRESCO campus of EBSU, Nigeria. A total of 82 E. coli isolates was isolated from the ready-to-eat foods and zobo drinks included in this study. On the other hand, 31 K. pneumoniae and 20 P. aeruginosa were also isolated from these food samples (Table 1). The antimicrobial susceptibility profile of the isolated bacteria from the ready-to-eat foods is shown in Table 2. Result of the antimicrobial sensitivity pattern of the isolates to different antibiotics showed that percentage susceptibility of E. coli; K. pneumoniae and P. aeruginosa isolates to the tested antibiotics were 83.33, 50 and 66.67%.

DISCUSSION

A handful of Nigerian students and other individuals depend mainly on food vendors, fast food centres and nearby restaurants that sell a variety of ready-to-eat foods (including jellof-rice, abacha, moi-moi, and zobo drinks) for their daily meal. A number of reasons abound for this rising development, but most importantly they patronize these fast food centres for want of time or sheer laziness in taking time out to cook the food themselves. As a result, they are at high risk of exposure to food-borne diseases due to poor handling and poor
preparation of these foods, a practice that allows pathogenic microorganisms to thrive in them and cause infection upon consumption. E. coli, K. pneumoniae and P. aeruginosa were the organisms isolated from ready-to-eat foods including zobo drinks sold around the PRESCO campus of EBSU, Abakaliki, Nigeria, but E. coli (a uropathogen that indicates fecal contamination) was the most prevalent bacteria isolated (Table 1). This was followed by K. pneumoniae and P. aeruginosa.

Studies both within and outside Nigeria have shown that E. coli and other enteric pathogens including K. pneumoniae and the non-enteric organism P. aeruginosa are responsible for many of the global cases of food poisoning (Ilediora et al., 2006; Muinde et al., 2005; Marwa et al., 2012). This is not far from the truth owing to the variety of bacteria isolated from the food samples in this study (Table 2). Lack of access to portable water and poor handling of foods in this area may have contributed to the worrisome frequency of pathogenic microbes in ready-to-eat foods and zobo drinks at the PRESCO campus of EBSU, Abakaliki, Nigeria. The antimicrobial susceptibility studies of the recovered bacterial isolates from ready-to-eat foods in this work to some selected antibiotics showed that the E. coli isolates were completely resistant to ampicillin, ofloxacin and clindamycin (Table 2). However, the isolate was susceptible to ciprofloxacin, erythromycin and gentamicin.

According to a recent report, multidrug resistance in E. coli strains from food origin was significantly higher than those from clinical origin, and this has been associated to the fecal source of the pathogen (Ochman et al., 2000). Fecal contamination of food portends danger to the health of those consuming them, owing to the notoriety of E. coli in multidrug resistant diseases. The percentage susceptibility of the bacterial isolates from the ready-to-eat foods and zobo drink revealed percentage susceptibilities of 66.7% (P. aeruginosa), 83.33% (E. coli) and 50% (K. pneumoniae) to the tested antibiotics. Frequency of K. pneumoniae, E. coli, and P. aeruginosa has also been reported from fermented zobo drinks in southwest Nigeria, and these are responsible for some of the food-borne illnesses in that region (Ojoko et al., 2002).

The presence of bacterial pathogens in the marketed zobo drinks is probably related to the source or quality of water used for their processing. The hawking of ready-to-eat foods and zobo drinks also predisposes them to dust particles which may harbour pathogens that lead to food poisoning upon consumption. The frequency of bacterial pathogens from food samples and their resistance to some available antibiotics as obtained in this study (Tables 2) is worrisome due to the notorious nature of the isolated pathogens (E. coli, K. pneumoniae and P. aeruginosa) in terms of drug resistance. Routine microbiological analysis of ready-to-eat foods including zobo drink around this region and other parts of Nigeria is paramount to curtail any disease outbreak in form of food poisoning due to these pathogens. Such practice if dutifully followed will ensure that quality foods are sold to unsuspecting customers, and the emergence and spread of resistant microbes through them will also be contained. Finally, this study revealed the presence of enteric and non-enteric organisms including E. coli, K. pneumoniae and P. Aeruginosa in the ready-to-eat food and zobo drink sold around the PRESCO campus of EBSU, Abakaliki, Nigeria, and the microbes are resistant to some available drugs. Regular monitoring of the quality of foods and drinks sold to students and other unsuspecting members of the public in this region is required to forestall

### Table 1. Distribution of isolated bacterial pathogens in the ready-to-eat food samples and zobo drinks.

<table>
<thead>
<tr>
<th>Food samples</th>
<th>Escherichia coli</th>
<th>Klebsiella pneumoniae</th>
<th>Pseudomonas aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice (n=50)</td>
<td>20</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Abacha (n=50)</td>
<td>15</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Mol-mol (n=50)</td>
<td>25</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Zobo drink (n=50)</td>
<td>22</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>31</td>
<td>20</td>
</tr>
</tbody>
</table>

### Table 2. Antibiotic susceptibility pattern of bacterial isolates from Zobo drink.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Zones of inhibition (mm)</th>
<th>% Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>E  CIP OFX CN AMP DA</td>
<td>4 (66.67)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>20 23 18 5 9 16</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>19 33 12 27 21 20 5</td>
<td>5 (83.33)</td>
</tr>
<tr>
<td></td>
<td>19 26 16 21 17 17 3</td>
<td>3 (50)</td>
</tr>
</tbody>
</table>

AMP = ampicillin, CIP = ciprofloxacin, OFX = ofloxacin, DA = clindamycin, E = erythromycin, CN = gentamicin.
any imminent health danger. Food handlers should also be educated and be observant of current public health guidelines in their profession so as to minimize food-borne related illnesses.

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Coco peat - An alternative artificial soil ingredient for the earthworm toxicity testing

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The artificial soil medium recommended for invertebrate toxicity studies by OECD and ISO contains sphagnum peat as an organic component. Sphagnum peat is not widely available in tropical countries especially in the Indian subcontinent. Using of native organic matter source is also much more ecologically relevant for the region. Hence, development of an alternative is needed as a replacement of sphagnum peat. As coco peat is easily available as an organic component in tropical countries, earthworm toxicity studies were conducted with coco peat to assess its suitability to be included as an alternative in the artificial soil medium. Artificial soils were prepared with 70% sand, 20% kaolin clay and 10% coco peat (COPS) or sphagnum peat (SPPS). Acute and reproduction toxicity studies were conducted with the earthworm, Eisenia fetida using 2-chloroacetamide and carbendazim, respectively. Validity criteria specified by the guidelines were met in tests with either soil media. In the acute test, no significant difference was observed between the soils in terms of mortality (p > 0.05) based on the LC₅₀ values for COPS and SPPS of 35.56 and 32.36 mg 2-chloroacetamide /kg dry soil, respectively. Significant effect in terms of reproduction was observed at 2.06 mg carbendazim/kg dry soil for both COPS and SPPS. The other parameters such as biomass change, mortality and food consumption were comparable.

Key words: Coco peat, shagnum peat, artificial soil, earthworm toxicity, carbendazim, 2-chloroacetamide.

INTRODUCTION

In terrestrial ecotoxicity assessment, especially with soil living organisms like earthworms, enchytraeids, and collembolans, the toxicity of a substance is strongly influenced by the medium (that is soil type and properties) in which the organisms are exposed. Hence, risk assessment for chemicals in soil has to take into account soil properties. This consideration led to the conclusion that it would be preferable to standardize the soils used for determining the toxicity of chemicals to soil organisms (P Mangala et al., 2009; Gawlik, 2001). In the artificial soil (suggested by the guidelines OECD, 1984; 2004; ISO 2012a:b), sphagnum peat is a component that represent soil organic matter. Among soil ingredients, peat consisted about 5 to 10% of the total formulation. The other soil ingredients are 69 – 74% sand, 20% clay and 0 to 1% CaCO₃ (to increase the pH up to 6.5).
Sphagnum peat is obtained from various species of the plant *Sphagnum*. Decayed, compacted *Sphagnum* moss also known as peat or peat moss is used as a soil conditioner. The cost of sphagnum peat is however increasing (Meerow, 1994) and there is a growing concern that it is mined from the endangered *Sphagnum* plant ecosystems which are declining rapidly due to environmental constraints (Barkham, 1993; Robertson, 1993; Frolking et al., 2001). However, recently, it has become evident that sphagnum peat is scarce and completely unavailable in many regions, including the tropics (P Mangala et al., 2009). Garcia (2011) and Römfbke et al. (2007) also considered this problem and suggested using locally available materials like coir dust, for performing soil ecotoxicity tests especially in these regions. Increasing peat-land conservation has also created a need to find possible alternatives. Coco peat also known as coir pith, coir fiber pith, coir dust, or simply coir, is made from coconut husks, a byproducts of industries that use coconuts. Consequently, coco peat is readily available in tropical countries.

Only few studies have been conducted with various organic materials including coco peat as a replacement to sphagnum peat in artificial soil (Noguera et al., 2000; Meerow, 1994; Abbiramy et al., 2012). However, acute and chronic earthworm studies with the chemicals suggested by OECD and ISO guideline are essential to validate the alternate soil so as to be included in the present OECD guideline.

The main objective of the study was to determine the suitability of composed coco peat as an alternative to sphagnum peat in the artificial soil used for earthworm toxicity studies. The soils used in the present studies are referred to as coco peat soil (COPS) and sphagnum peat soil (SPPS). The validation of the COPS was tested in comparison with SPPS using the standard chemicals, such as 2-Chloroacetamide and Carbendazim suggested by OECD guidelines for acute and chronic exposure of earthworms respectively. Comparison of the physico-chemical parameters of the two peat samples was also studied.

**Preparation of artificial soils**

The modified artificial soils were prepared as described in the OECD guideline (1984) for sphagnum and composted coco peat, separately. Sphagnum peat imported from Gramoflor GmbH & Co Germany as solid blocks, was shade dried and powdered by grinding machine with a particle size of <2 mm using sieves. Similarly, the composted coco peat obtained from Varsha enterprises, Bangalore, India, was also shade dried and pulverised with the same particle size of <2 mm. The other soil ingredient, kaolin clay was purchased from Romac India ltd, Chennai, India. River sand was used in place of quartz sand with a particle size of approximately 50 to 200 microns. The artificial soil was prepared by mixing 70% sand, 20% clay and 10% peat in as dried constituents in a laboratory homogeniser for 20 min and stored in an air tight plastic container at room temperature for the coco and sphagnum peat samples separately. As the pH of both artificial soils were determined to exceed the range specified by the OECD guidelines (6 ± 0.5), CaCO₃ was not needed to increase the pH.

Similarly, for reproduction test, the artificial soil was prepared with the ratio of 69% sand, 20% clay, and 10% peats. Remaining 1% of sand was added along with test substance since the test item, Carbendazim (98% pure) was insoluble in any of the solvent. pH of the artificial soil samples with cow manure powder (at 1% total weight) was checked and found that these exceeded the range specified by the guideline (ISO, 1994) (6 ± 0.5). For both the peat based soils, CaCO₃ was not needed to increase the pH.

The Maximum Water Holding Capacity (MWHC) of each soil samples were done by the method specified in the guideline (OECD, 2004) with slight modifications. Approximately, 50% of the MWHC was used to moisten the soil for the earthworm survival in the soils for both acute and chronic tests.

**Evaluation of earthworm toxicity**

Toxicity studies included both acute and chronic using the test chemicals suggested by OECD and ISO guidelines. The validation of the soils were done via acute and reproduction (chronic) toxicity tests to the earthworm, *Eisenia fetida*, obtained from the culture maintained in the Department of Ecotoxicology, International Institute of Biotechnology and Toxicology (IIBAT). The test chemical suggested by the guidelines (OECD, 1984; ISO, 1993), namely, 2-Chloroacetamide (100% pure, Sigma Aldrich, Netherlands) and Carbendazim (>97% pure, Sigma Aldrich, Netherlands) as suggested by the guideline were used to validate the soils for acute and chronic tests, respectively.

**Acute toxicity test**

Glass beakers of 1 L capacity with a cross sectional areas of 113 cm² were used in the experiments. For each replicates, about 550±10 g of dry artificial soil was filled into the beaker. The dried artificial soil was moistened to approximately half of the final water content one day before the application to avoid the dust emission during test compound application. Five treatment of 2-Chloroacetamide: 24, 30, 38, 47 and 59 mg/kg dry soil and an untreated control (moistened with deionised water) were prepared separately for each soil type. The concentrations were selected based on a preliminary range finding test. The respective amounts of test chemical were weighed in separate glass vials, dispersed in a determined quantity of deionised water (about 30 ml/100 g dry soil), applied to the artificial soil and homogenised using a laboratory mixer. Healthy earthworms (4 to 6 months old with a wet weight of 350 to 550 mg/worm (with gut content) with well-developed clitellum which were acclimated for one day in the respective soils were selected, washed with tap water, blotted...
carefully with filter paper, weighed and released on the surface of the treated artificial soil. Four replicate for each concentrations and control were used, with ten earthworms in each replicate. The test containers were incubated in a temperature controlled room at 20±2°C and 400 to 800 lux continuous light on the test containers for 14 days. The pH of the artificial soil at start of the test was 6.67 and 6.80 for sphagnum peat soil (COPS) and coco peat soil (SPPS), respectively.

After 7 and 14 days of exposure, the artificial soils were removed from the containers and the earthworms were counted as live or dead. Due to rapid decomposition in the soil, the missing earthworms were considered as dead. After the mortality check on day 7, the live earthworms and the artificial soil were returned to the respective test containers. The total and the mean body weights of all live earthworms in each test container were determined at the test start (day 0) and end (day 14). Based on the weight difference between initial and final weight, the biomass change was calculated. At the end of 14 days, moisture content and pH of the artificial soils were assessed.

**Reproduction (Chronic) toxicity test**

Glass beakers of 2 L capacity with 150 cm² cross sectional area were used for reproduction test. For each replicate, about 500±10 g of dry artificial soil was filled into the beaker. Pre moistening of artificial soil was done with approximately half of the final water content one day before the application to avoid the dust emission during test compound application. On the day of experiment, the remaining water was added along with test item-quartz sand mixture. Based on a preliminary 14 days acute range finding test results, nine treatment concentrations of Carbedazim, namely, 0.2, 0.35, 0.64, 1.14, 2.06, 3.70, 6.67 and 12.00 mg/kg dry soil were prepared separately for each soil type and a control (moistened with deionised water). The respective amounts of test chemical were weighed in separate glass vials. Since the test item was not soluble in solvents, it was dispersed in 5 g of quartz sand for each concentration separately, applied to the artificial soil, and homogenised using a laboratory mixer. Healthy earthworms (4 to 6 months old with a wet weight of 280 to 490 mg/worm including gut contents) with well-developed citellum were acclimated for one day in the respective soils. During acclimatisation, powdered wet cow manure was added on the top of the soil. Ten earthworms were added to each of the four replicates of treated soil and to each of the eight replicates of control soil. The test containers were incubated at controlled room temperature controlled room at 20±2°C. The light intensity of 400 to 800 lux with 16:8 h light:dark cycle was maintained.

Adult earthworms were exposed to treated soil for 4 weeks. After 4 weeks, adults were sorted from the soil and observed for mortality. The live earthworms were weighed and discarded. The soils of each treatment replicate was then returned to the respective containers and incubated under the same test conditions for additional 4 weeks for reproduction assessment.

After a total of 8 weeks (56 days), the number of juveniles in each container was determined. Juveniles were collected from the soil manually by sorting the soil in enamel tray then the tray was placed in a water bath at approximately 50°C. Juveniles which wriggle out from the soil were counted. Soil was then sorted again manually to recover the remaining juveniles if any. Soil pH and soil water content were determined for each treatment group at start and end of the test.

Cow manure from clinically normal cows was collected, shade dried and powdered for use as food for the earthworms. One day after test item application, 12 g of feed (5 g of finely ground cow manure was mixed with 7 g deionised water) was scattered uniformly on the soil surface in each container. Observation of feed consumption was made visually and based on the previous week consumption; the fresh feed was added in the same way for the first four weeks of the experiment (that is, days 1, 8, 15 and 22). After removal of the adult worms on day 28, cow manure (5 g/container) was carefully mixed into the artificial soil to feed the juveniles. No additional food was given thereafter.

**Statistical analysis**

All the statistical analyses were carried out with TOXSTAT version 3.5 software. Statistical tests used for the study are thus:

1. For the acute study, the parameters and the statistical tests used are as follows:
   a. LC₅₀ - Probit analysis (Finney, 1971)
   b. NOEC based on Biomass – Dunnett’s test (multiple comparison, one-sided, (α = 0.05))
   c. NOEC based on Mortality Fisher’s exact test (α = 0.05)
   d. Comparison toxicity level of soils: Two factorial ANOVA
   e. Comparative physico-chemical parameters: Student’s t test

2. For the chronic study, the parameters and the statistical tests used are as follows:
   a. LC₅₀ and EC₅₀ - Probit analysis (Finney, 1971)
   b. NOEC based on Biomass and reproduction-Bonferroni test (multiple comparison, one-sided, α = 0.05) and Wilcoxon’s Rank Sum Test
   c. NOEC based on Mortality-Fisher’s exact test (α = 0.05)
   d. Comparison of soils: Two factorial ANOVA
   e. Comparative physico-chemical parameters: Student’s t test

**RESULTS**

**Physico-chemical parameters of peats**

The physico-chemical properties such as pH (CIPAC MT-75), total organic carbon and total organic matter (Jackson, 1973), phosphorus, calcium, magnesium (Sundaram et al., 2001), nitrogen (Van soest, 1975), potassium (AOAC, 1956), water holding capacity and bulk density of composted sphagnum and coco peat are as shown in Table 1. Physico-chemical properties differed significantly between the peat samples. Water holding capacity, organic carbon and total organic matters were significantly higher in sphagnum peat as compared to coco peat. However, the macro nutrients (N, P, K) and micro nutrients (Calcium and Magnesium) were significantly higher in coco peat than in sphagnum peat.

**Acute effect**

The acute effect of 2-Chloroacetamide on earthworm, E. fetida is shown in Table 2. Significant mortality was observed at 30 mg a.i./kg dry soil and greater concentrations, in either COPS or SPPS, compared with their respective controls. Complete mortality was observed in 59 mg a.i./kg dry soil, the highest concentration in both soils tested. The LC₅₀ values of 35.56 and 32.36 mg a.i./kg dry soils were determined for COPS and SPPS,
Table 1. Physico-chemical properties of composted sphagnum and coco peat.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sphagnum peat</th>
<th>Coco peat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water holding capacity (%)</td>
<td>540.85</td>
<td>481.61*</td>
</tr>
<tr>
<td></td>
<td>(14.03)</td>
<td>(6.71)</td>
</tr>
<tr>
<td>Bulk density (g/mL)</td>
<td>0.143</td>
<td>0.193*</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>pH (in 0.01M CaCl₂)</td>
<td>3.62</td>
<td>4.74*</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.03)</td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>47.19</td>
<td>38.04*</td>
</tr>
<tr>
<td></td>
<td>(0.76)</td>
<td>(0.42)</td>
</tr>
<tr>
<td>Total organic matters (%)</td>
<td>98.62</td>
<td>89.09*</td>
</tr>
<tr>
<td></td>
<td>(1.21)</td>
<td>(1.02)</td>
</tr>
<tr>
<td>N (mg/kg)</td>
<td>1250</td>
<td>1380*</td>
</tr>
<tr>
<td></td>
<td>(7.61)</td>
<td>(4.55)</td>
</tr>
<tr>
<td>P (mg/kg)</td>
<td>125</td>
<td>350*</td>
</tr>
<tr>
<td></td>
<td>(1.22)</td>
<td>(2.79)</td>
</tr>
<tr>
<td>K (mg/kg)</td>
<td>5788</td>
<td>6490*</td>
</tr>
<tr>
<td></td>
<td>(12.58)</td>
<td>(15.28)</td>
</tr>
<tr>
<td>Ca (mg/kg)</td>
<td>92500</td>
<td>97500*</td>
</tr>
<tr>
<td></td>
<td>(301.4)</td>
<td>(300.0)</td>
</tr>
<tr>
<td>Mg (mg/kg)</td>
<td>1500</td>
<td>2500*</td>
</tr>
<tr>
<td></td>
<td>(4.68)</td>
<td>(7.99)</td>
</tr>
</tbody>
</table>

*Significantly different from sphagnum peat (t test α=0.05). P = 0.024. Figures in parentheses are standard deviation.

respectively (With 95% confidence interval of 34.18 to 37.02 and 30.85 to 33.80 mg a.i./kg dry soil for COPS and SPPS, respectively).

Significant biomass change from control was observed from the concentration, 30 mg a.i./kg dry soil in both SPSS and COPS. The NOEC related to biomass was observed at 24 mg 2-Chloroacetamide mg/kg dry soil for both COPS and SPPS. The overall comparisons on biomass change between the soils were not significant (Two-factorial ANOVA, p>0.05).

Reproduction effect

The effect of Carbendazim on mortality and biomass change of *E. fetida* is shown in Table 3. No mortality was observed up to the concentration of 2.06 mg a.i./kg dry soil for both soils tested. Significant mortality was observed from the concentration of 6.67 mg a.i./kg dry soil for both COPS and SPPS. The LC50 of Carbendazim at 28 days after exposure was observed as 6.72 and 6.33 mg a.i./kg dry soil for COPS and SPPS, respectively (With 95% confidence interval of 6.06 to 7.38 and 5.85 to 6.80 mg a.i./kg dry soil for COPS and SPPS, respectively). The NOEC and LOEC values related to mortality were found to be 3.70 and 6.67 mg a.i./kg dry soil, respectively for both the soils (Fisher’s exact test). The overall comparison at all the dose response level of the soils on mortality was not significant (Two-factorial ANOVA, p = 0.05).

The biomass of adult earthworms exposed for 28 days showed an increase in both the controls of COPS (+27.26%) and SPPS (+32.01%). Significant biomass change from control was observed from the concentration, 2.06 and 3.70 mg a.i./kg dry soil in COPS and SPPS, respectively. Hence, the NOEC related to biomass was found to be 1.14 and 2.06 mg a.i./kg dry soil for COPS and SPPS, respectively (Dunnett test). The
Table 2. Acute effect of 2-chloroacetamide on the earthworm, *Eisenia fetida* in formulated soils using coco peat and sphagnum peat.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Soil type</th>
<th>Control (deionised water)</th>
<th>2-chloroacetamide (mg a.i./kg dry soil)</th>
<th>Statistical comparison between soils (P value)&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>Mortality after 14 days (%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>COPS</td>
<td>0</td>
<td>0&lt;sup&gt;n.s&lt;/sup&gt; (-)</td>
<td>25.00* (5.8)</td>
</tr>
<tr>
<td></td>
<td>SPPS</td>
<td>0</td>
<td>5.00&lt;sup&gt;n.s&lt;/sup&gt; (5.8)</td>
<td>40.00* (8.2)</td>
</tr>
<tr>
<td>Biomass change after 14 days (%)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>COPS</td>
<td>-13.81 (4.65)</td>
<td>-19.70 (6.5)</td>
<td>-20.09* (12.9)</td>
</tr>
<tr>
<td></td>
<td>SPPS</td>
<td>-12.53 (2.38)</td>
<td>-22.41* (3.3)</td>
<td>-29.52* (5.4)</td>
</tr>
</tbody>
</table>

Mean of four replications; Figures in parentheses are standard deviation; n.s. = not significantly different compared to control; <sup>1</sup>= significantly different compared to control; <sup>2</sup>Fisher’s exact test (α = 0.05); <sup>3</sup>Dunnett test (α = 0.05) for SPPS and Steel’s many one rank test (α = 0.05) for COPS; <sup>3</sup>Two-factorial ANOVA.


<table>
<thead>
<tr>
<th>Carbendazim concentrations (mg a.i./kg dry soil)</th>
<th>COPS Mortality (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>SPPS Mortality (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>COPS Biomass change (%)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SPPS Biomass change (%)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
<td>+27.26 (5.2)</td>
<td>+32.01 (6.4)</td>
</tr>
<tr>
<td>0.20</td>
<td>0.0</td>
<td>0.0</td>
<td>+29.69&lt;sup&gt;n.s&lt;/sup&gt; (9.1)</td>
<td>+37.46&lt;sup&gt;n.s&lt;/sup&gt; (4.2)</td>
</tr>
<tr>
<td>0.35</td>
<td>0.0</td>
<td>0.0</td>
<td>+30.82&lt;sup&gt;n.s&lt;/sup&gt; (2.2)</td>
<td>+37.39&lt;sup&gt;n.s&lt;/sup&gt; (5.1)</td>
</tr>
<tr>
<td>0.64</td>
<td>0.0</td>
<td>0.0</td>
<td>+31.26&lt;sup&gt;n.s&lt;/sup&gt; (6.8)</td>
<td>+20.04&lt;sup&gt;n.s&lt;/sup&gt; (5.5)</td>
</tr>
<tr>
<td>1.14</td>
<td>0.0</td>
<td>0.0</td>
<td>+31.53&lt;sup&gt;n.s&lt;/sup&gt; (4.9)</td>
<td>+37.00&lt;sup&gt;n.s&lt;/sup&gt; (3.0)</td>
</tr>
<tr>
<td>2.06</td>
<td>0.0</td>
<td>0.0</td>
<td>+11.96* (1.1)</td>
<td>+23.71&lt;sup&gt;n.s&lt;/sup&gt; (10.5)</td>
</tr>
<tr>
<td>3.70</td>
<td>2.50 (5.0)</td>
<td>2.50 (5.0)</td>
<td>-30.11* (3.1)</td>
<td>-39.76* (5.5)</td>
</tr>
<tr>
<td>6.67</td>
<td>67.50* (9.6)</td>
<td>80.00* (8.2)</td>
<td>-52.51* (4.0)</td>
<td>-60.72* (17.8)</td>
</tr>
<tr>
<td>12.00</td>
<td>95.00* (10.0)</td>
<td>-100*</td>
<td>-61.76* (3.5)</td>
<td>-100*</td>
</tr>
</tbody>
</table>

Statistical comparisons between soil<sup>3</sup>

<table>
<thead>
<tr>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0608&lt;sup&gt;n.s&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean of four replications; figures in parentheses are standard deviation, n.s. = not significantly different; * = significantly different. <sup>1</sup>Fisher’s exact test (α = 0.05); <sup>2</sup>Bonferroni test (α = 0.05); <sup>3</sup>Two-factorial ANOVA.

Overall dose response biomass change for both soils were not significant (Two-factorial ANOVA, α = 0.05).

The reduction in reproduction for the concentration of carbendazim over control is shown in Figure 1. The mean number of juveniles produced was found to range from 0 to 92 and 0 to 87.5 in coco peat and sphagnum peat treated soils, respectively (Figure 2). The significant effect on reproduction was observed at 2.06 mg a.i./kg dry soil for both COPS and SPPS. However, the effective concentration based on reproduction...
(EC\textsubscript{50}) of COPS and SPPS was found to be 1.35 and 2.45 mg a.i./kg dry soil, respectively.

**DISCUSSION**

In a toxicity study with earthworm, the nature of the soil medium is a crucial role to decide the toxicity of the xenobiotics on earthworms despite other factors like temperature, light intensity, etc. In the present study, the test medium which was compared under similar experiment conditions were identical in all aspects but for the peat. Hence, the reason for the variable endpoints is attributed to the variation in the soil ingredient, especially the organic content, peat.

Analysis of physico-chemical properties of the biological materials resulted in significant variation. P Mangala et al. (2009) studied similar physico-chemical properties in the prepared artificial soil. The pH of the sphagnum peat was found to be significantly acetic when compared to coco peat which was in line with reports of Geoff (2011). Though the pH of the peat samples was found significantly different, there was no significant variation between artificial soil samples.
Water holding capacity of sphagnum peat was significantly higher than that of coco peat. Similarly, the moisture content of the two soils during the acute and chronic experiment varied from 30.07 to 32.66% and 32.81 to 37.76% for COPS and SPPS treated soils, respectively. The moisture content is also influencing toxicity of certain compounds (Puurtinen and Martikainen, 1997). In this study, there was no appreciable variation in moisture content of the two soils, indicating that the moisture content is not influencing toxicity. The total organic matters play an important role to predict biological response of earthworms in Cu contaminated soil (Gonzalo, 2009). The concentration of essential elements such as NPK, Mg and Ca was significantly higher in coco peat than sphagnum peat samples. Manuel Abad et al. (2005) studied significant variation in the physico-chemical properties of coco peat with different locations in which they are growing. The variation of physico-chemical properties with location is not only applicable for coco peat but also for the sphagnum peat. Abbiramy et al. (2012) also observed similar physico-chemical characteristics in the two peat samples. This study is also in line with their findings, except for few properties. They validated the soil with coco peat using urea as substance.

The validity criteria such as control mortality (≤ 10%) and mean weight loss in control (≤ 20%) for acute earthworm toxicity test were met in both the soils tested (Organisation for Economic Co-operation and Development (OECD), 1984; International Organisation for Standardisation (ISO), 2012). As per the ISO guideline (ISO, 2012), the LC50 of the reference compound, 2-chloroacetamide should be 20 to 80 mg a.s/kg dry soil. This value is specified to validate the test condition, especially for the exposing medium (artificial soil). The LC50 of 2-chloroacetamide for SPPS and COPS was well within the range specified by the guideline. Hence, both the soil met the validity criteria specified by the guideline. Though the LC50 of 2-chloroacetamide was found to be within the validity range, the comparative analysis on mortality revealed that there was a significant difference between the soils (two-factorial analysis of variance (ANOVA), p < 0.05). This may be due to the variation in the soils properties. However, the difference in the soil properties was not influenced in biomass change. Therefore the 14 days acute test on earthworm with coco peat is comparable with sphagnum peat. Though the soil components differed between soils, mortality of carbendazim between the soils was not significant, which is in line with Sian et al. (2007) who reported that the soil components did not greatly influence carbendazim toxicity to E. fetida.

The number of juveniles in control ranged from 70 to 102 in COPS and 72 to 128 in SPPS. Though there was a significant variation in number of juveniles produced between the soils, both of them met the validity criteria specified by the guideline. The coefficient of variation also met the guideline requirement which was observed to be 14.32% in COPS against SPPS (17.85%). The EC50 of carbendazim showed that there is no difference between the two soil types tested (1.35 and 2.45 mg a.s/kg dry soil for COPS and SPPS, respectively). However the EC50 value of carbendazim reported by P Mangala et al. (2009) for coco peat and sphagnum peat soil was observed as 1.2 and 1.0 mg a.s/kg dry soil, respectively.

Conclusions

The following conclusions can be drawn based on the results of the study:

1. Peat material, the only organic ingredient in the artificial soil medium, plays a major role in determination of toxicity level in earthworm toxicity testing. 2. Physico-chemical properties of peat material do not significantly influence earthworm toxicity.
3. Composted coco peat is a good organic source for the replacement of sphagnum peat in artificial soil preparations.
4. The alternate artificial soil prepared using coco peat can be used to perform earthworm toxicity studies (both acute and chronic), especially in tropical countries.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. A. Ramesh, Head, Department of Analytical Chemistry, IIBAT for providing facility to analyse the physico chemical properties of peat samples. The authors also appreciate Mr. R. Radhakrishnan, Scientist, Department of Analytical Chemistry and Ms. S. Hilda, Scientist, Department of Ecotoxicology IIBAT for their useful contribution to fulfill this paper work.

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