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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Microbiology Research

Table of Content: Volume 10 Number 8, 28 February, 2016

ARTICLES

Detection of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of Klebsiella pneumoniae using the ESBL NDP test and flow cytometric assay in comparison to the standard disc diffusion 238
Samaa Taha, Nahed Youssef, Amany Elkazaz and Hazem Ramadan

Physicochemical and in vitro antimicrobial activity of the oils and soap of the seed and peel of Citrus sinensis 245
Minda Asfaw Geresu, Gobena Ameni, Tesfu kassa, Getachew Tuli,

The growth potential and antimicrobial susceptibility patterns of Salmonella species and Staphylococcus aureus isolated from mobile phones of food handlers and health care workers in Jimma Town, Southwest Ethiopia 254
Tsegaye Shamebo, Ketema Bacha and Tsige Ketema

Anti-bacterial, anti-oxidant and cytotoxicity of aqueous and organic extracts of Ricinus communis 260
Shazia Mansoor, Imran Khan, Jasmine Fatima, Mohd Saeed and Huma Mustafa

academicJournals

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Full Length Research Paper

Detection of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* using the ESBL NDP test and flow cytometric assay in comparison to the standard disc diffusion

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This study was undertaken to evaluate the comparison among three different assays: extended-spectrum beta-lactamases (ESBL) Nordmann/ Dortet/ Poirel (NDP) test, flow cytometric assay and disc diffusion method for the detection of ESBL production. Sixty clinical isolates of *Klebsiella pneumoniae* were isolated from patients' clinical samples admitted to Suez-Canal University Hospital, Ismailia Governorate. The percentages of ESBLs producing *Klebsiella pneumoniae* ranged from 70 to 80% by ESBL NDP and flow cytometric assays, respectively in comparison to 76.6% by disc diffusion method. The sensitivity and specificity of the three assays were evaluated and the sensitivity by ESBL NDP and disc diffusion method was 100%, while by the flow cytometric assay, it was 91.3%. The specificity of disc diffusion method in detection of ESBLs was 100%, followed by the ESBL NDP test (85.7%) and flow cytometric assay (77.8%). Kappa testing showed perfect agreement between the ESBL NDP test and disc diffusion method (kappa=0.9), while flow cytometric assay showed substantial agreement (kappa= 0.7). The ESBL NDP test offers an applicable tool for rapid detection of ESBL-production. Although, flow cytometric assay is a promising method that might be used in the clinical microbiology laboratory but there is a need for the experienced personnel along with the device.

Key words: Extended-spectrum beta-lactamases (ESBLs), ESBL NDP test, flow cytometry.

INTRODUCTION

Extended-spectrum beta-lactamases (ESBLs) produced by Gram-negative bacteria are considered one of the largest and rapidly evolving group of plasmid-mediated enzymes that confer resistance to oxyimino-cephalosporins and monobactams (Pitout, 2010). *Escherichia coli* and *Klebsiella pneumoniae*, being the major source of community- and hospital-acquired infections are mostly ESBL producers (Pitout and Laupland, 2008).

ESBL recognition has an important clinical impact as

inappropriate treatment can lead to therapeutic failures and consequently to adverse clinical outcomes (Schwaber and Carmeli, 2007). A variety of ESBLs have been reported in *Enterobacteriaceae*, being mostly of the CTX-M-, TEM- and SHV-types (Bush and Jacoby, 2010; Poirel et al., 2012). ESBL detection is necessary to screen patients, improve hospital infection control practices and to curb inappropriate antibiotic used that prolonged the efficacy of the currently available antibiotics (Schwaber et al., 2006; Zahar et al., 2009).

Current techniques for detecting ESBL producers are based on the determination of susceptibility to expanded-spectrum cephalosporins followed by the inhibition of the ESBL activity, mostly by clavulanic acid or tazobactam (Drieux et al., 2008). Sensitivities and specificities of the double disk test and of the E-test proposed for that purpose are good, ranging from 80 to 95% (Gazin et al., 2012). The automated methods used in the detection of ESBL producing organisms had a much higher sensitivity (80 to 99%) than specificity (50 to 80%). However, those tests require overnight growths consuming 24-48 h before ESBL production is detected with a subsequent delay in the initiation of appropriate antibiotic therapy (Schwaber et al., 2006; Drieux et al., 2008; Gazin et al., 2012).

Molecular detection of ESBL genes (PCR and sequencing) is an interesting alternative but remains costly and requires a certain degree of expertise (Drieux et al., 2008; Gazin et al., 2012) since recently, real time PCR and DNA microarray (Check-Points) are commercially available to detect ESBL gene variants (Cuzon et al., 2012). However, those PCR-based techniques require isolation of bacteria from clinical samples prior to susceptibility testing and phenotypic identifications and hence; those results can be obtained at least 48 h after obtaining the clinical samples. Also, they are usually not performed in a routine laboratory but restricted to epidemiological purposes. Therefore a simple and efficient technique for ESBL producers is required (Nordmann et al., 2012).

The ESBL NDP test is a novel test, based on the hydrolysis of the β-lactam ring of a cephalosporin (cefotaxime), which generates a carboxyl group, by acidifying a culture medium. It uses 96-well microtiter plates or a single tube and the acidity resulted from this hydrolysis is identified by the color change using a pH indicator (red phenol) while, inhibition of ESBL activity is evidenced by adding tazobactam in a complementary well (Cuzon et al., 2012).

A rapid, powerful high-throughput technology allowing analysis of several thousand cells per second and providing quantitative and statistically significant data is the flow cytometry (FC) (Shapiro, 2001). Bacterial cells are incubated with cephalosporins (ceftazidime or cefotaxime) in the presence and absence of clavulanic acid; subsequently, cells are stained with the fluorescent dye Bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3)] which is able to diffuse across depolarized membranes. Susceptible isolates display increased fluorescence after 1 h of incubation; conversely, the increase of the depolarized population was only observed after incubation with clavulanic acid associated with ceftazidime or cefotaxime in ESBL producers (Ramos et al., 2012).

In the present study, two new methods (a flow cytometric assay and the ESBL NDP test) were assessed for detection of ESBLs in clinical isolates of *Klebsiella pneumoniae* in comparison with the standard disc diffusion method.

MATERIALS AND METHODS

Bacterial strains

A total of 60 clinical isolates of *K. pneumoniae* were isolated from patients (24 males and 36 females) with different clinical infections (12 sputum, 26 urine, 12 pus and 10 blood samples) admitted to Suez-Canal University Hospital, Ismailia Governorate from January to August 2014. The samples were collected from various clinical origins. Blood samples were inoculated into blood culture bottles (Egyptian Diagnostic Media, Egypt) then incubated at 37°C for 7-14 days. Subcultures were done every 48 h on blood agar and MacConkey's agar (Oxoid, UK) plates. Other samples were cultured on nutrient agar (Oxoid, UK) blood agar and MacConkey's agar. Gram negative bacilli giving non-lactose fermenting colonies on MacConkey's agar were taken for biochemical test including mannitol motility, triple sugar ion, indole, citrate, MR, VP and carbohydrate utilization tests for identification (Birgul, 2010). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as ESBL- positive and negative, respectively (CLSI, 2014). All isolates were kept in soft agar at -20^{0}C till the time for ESBL detection.

Antimicrobial drugs and ESBL phenotypic detection

For the disc diffusion method, antibiotic discs of ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), Cefotaxime- clavulanic acid (30/10 µg) (CTC 40 µg) and Ceftazidime- clavulanic acid (30/10 µg) (CZC 40 µg) were purchased from Bioanalyse Chemical Co Ltd, Turkey. Cefotaxime sodium salt, tazobactam (TZB) and clavulonic acid (CLA) were purchased from Sigma-Aldrich, Saint-Quentin-Fallavier, France for the ESBL NDP test. For flow cytometric assay, bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3)], a fluorescent probe that binds to membranes and to intracellular proteins of depolarized cells, was purchased from Invitrogen/Life technologies, Carlsbad, USA; a stock solution (1 mg/ml) was prepared in dimethyl sulphoxide (DMSO).

The disc diffusion method

Stored isolates were subcultured on MacConkey's agar and the

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Figure 1. Representative results of the ESBL NDP test. Strains 1 and 2 are negative and positive controls, respectively; strains 3, 5,6,7,8 and 11 are ESBL producers; strains 4,9,10 and 12 are non-ESBL producers.

pure isolated colonies of identified bacteria was adjusted to 0.5 McFarland turbidity standards in 0.85% saline and lawn culture was spread using sterile swabs on Muller Hinton Agar media (Hi-media). All the strains were screened for ESBL production using CTX (30 µg) and CAZ (30 µg). Strains showing zone of inhibition of ≤ 27 mm for CTX and ≤ 22 mm for CAZ were selected for ESBL combined disc conformation test. Combined discs of CTC (40 µg) and CZC (40 µg) were used in the confirmation test according to the CLSI M2-A10 protocol (CLSI, 2009).

The ESBL NDP (Nordmann/ Dortet/ Poirel) test

Strains were isolated on MacConkey's agar and incubated at 37°C for 24 h before performing the NDP rapid ESBL test as described by Nordmann et al. (2012). Briefly, one calibrated loop inoculum (10 µl) of the tested strain was resuspended in 150 µl of 20 mM Tris-HCl lysis buffer in eppendorf tubes containing microbeads. Then, microbead tubes were vortexed for 30 min at room temperature for the mechanical lysis of bacteria. After centrifugation, 30 µl of the supernatant was mixed in a well of a 96-well tray with 100 µl of a 1 ml solution made of 3 mg of purified cefotaxime sodium salt in a pH 7.8 phenol red solution. The pH value was then adjusted to a 7.8 value by the addition of drops from 1 N NaOH solution. Mixture of the phenol red solution and the enzymatic suspension being tested was incubated at 37°C for 30 min. Similarly, culture extracts were analyzed in wells containing cefotaxime and tazobactam (4 mg/ml). A test was considered as positive when the well containing cefotaxime alone turned from red to yellow/orange and the well containing cefotaxime suplemented with tazobactam remained red (ESBL producer).

Flow cytometric analysis

Bacterial isolates from fresh agar plates were inoculated in trypticase soy broth and incubated at 37□C with shaking until the log phase was reached (about 1 h and 15 min). Subsequently, a suspension containing 5 x 10^6 cells/ml in fresh medium was prepared and the bacterial cells were exposed either to 4 mg/L of CTX, or 16 mg/L of CAZ, alone or with 4 mg/L of CLA, for 60 and 120 min. In parallel, after incubation, the cells were centrifuged and washed in PBS. The dye DiBAC4 (3) was added in a concentration of 1 µg/ml for 30 min, at room temperature and protected from light. The flow cytometric assay was used according to Ramos et al. (2012). It was performed on a FACSCalibur flow cytometer (BD, Sparks, USA). Nearly, 10,000–30,000 events of each sample were measured with the Software Cell Quest. The acquisition settings were defined using non-treated, non-stained cells (autofluorescence) and after adjusting the photomultiplier tubes' voltage to the first logarithmic (log) decade. The fluorescence intensity at 530/30 nm (FL1) was registered after incubation with antimicrobials and staining with 1 µg/ml DiBAC4 (3).

Statistical analysis

Sensitivity, specificity, positive and negative predictive values were assessed for the ESBL NDP test and the flow cytometric assay considering the standard disc diffusion method as a gold standard. The kappa values were calculated to evaluate the agreement between each of the ESBL NDP test and the flow cytometric assay and the disc diffusion method (Viera and Garrett, 2005).

RESULTS

The disc diffusion method had classified the 60 tested strains into 46 (76.6%) ESBL producers and 14 (23.3%) non- ESBL producers. Using the disc diffusion method, an ESBL producer isolates showed resistance to CTX and CAZ then the susceptibility increased (≥5 mm increase in zone diameter) to combined discs CTC and CZC while non- ESBL producer isolates were resistant to CTX and CAZ with no increase in the susceptibility to combined discs CTC and CZC.

Using the ESBL NDP test, 80% (n= 48) of the tested isolates produced ESBLs as the color of the wells turned from red to yellow in presence of cefotaxime and remained red when tazobactam was added (Figure 1) and 20% (n=12) tested negative for ESBL production. The sensitivity and specificity of the test were 100 and 85.7%, respectively in comparison with the standard disc diffusion method whereas the positive and negative

Table 1. Results of the disc diffusion method, the ESBL NDP test and the flow cytometric assay for detection of ESBLs in clinical isolates of *Klebsiella pneumoniae*.

Test result	The disc diffusion method	The ESBL NDP test	Flow cytometric assay
ESBL producers	46	48	42
Non- ESBL producers	14	12	18
Total	60	60	60
Sensitivity	100%	100%	91.3%
Specificity	100%	85.7%	77.8%
Positive predictive value		95.8%	91.3%
Negative predictive value		100%	77.8%

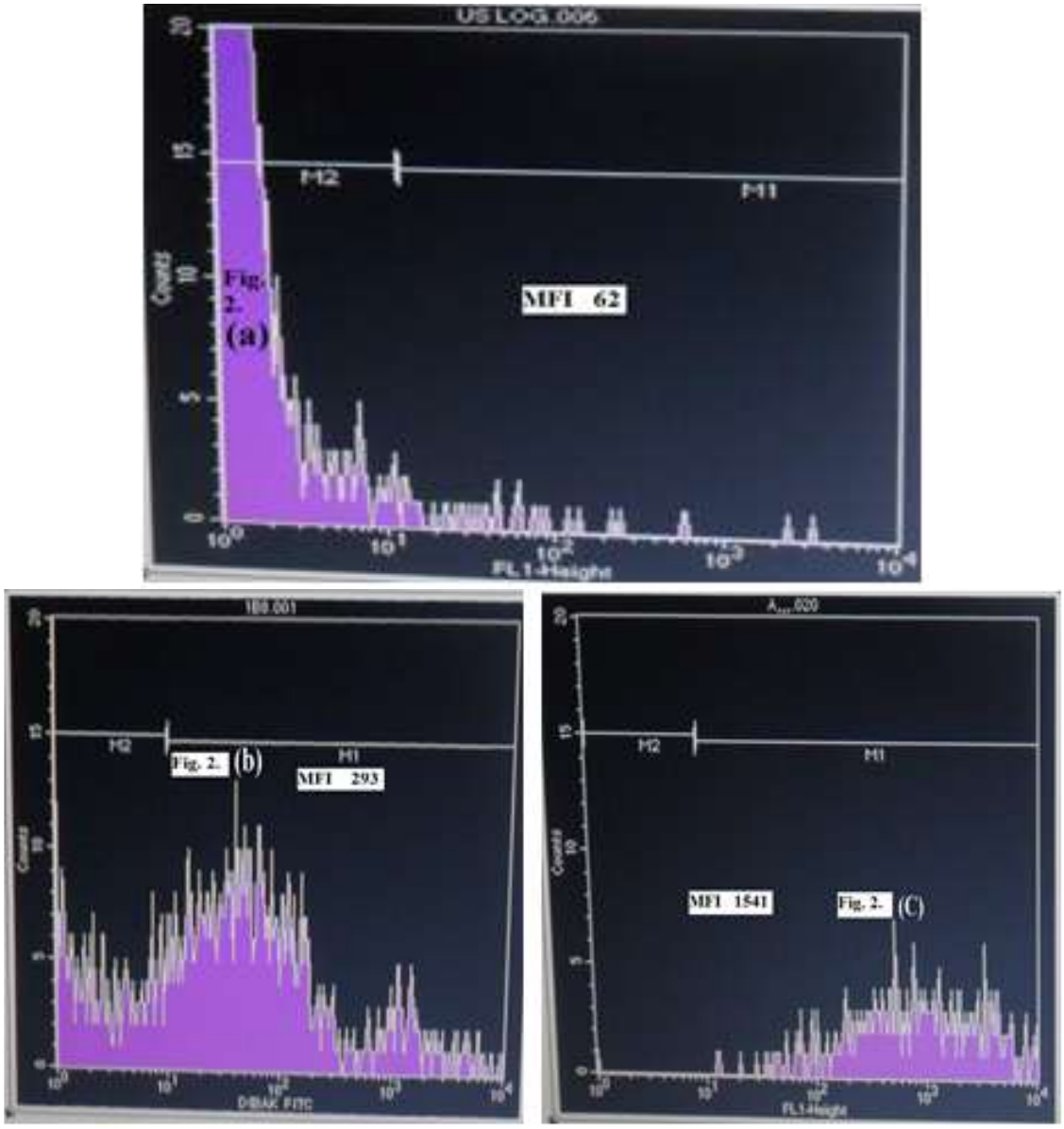

Figure 2. Flow cytometric histogram representing the emitted fluorescence at FL1 (green- 530 nm) of (a) non-treated and non-stained cells (autofluorescence) with mean fluorescence intensity (MFI) of 62 (b) an example of ESBL producer isolate after treatment with CTX (4 mg/L) for 60 min; the MFI was 293, and (c) after treatment with CTX (4 mg/L) and CLA (4 mg/L) for 60 min: the MFI was 1541.

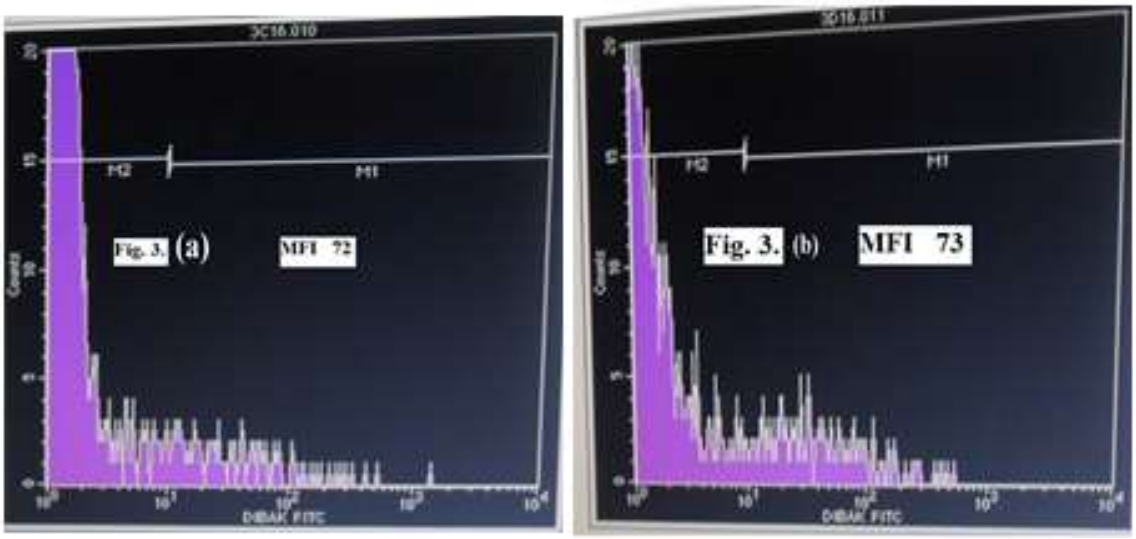

Figure 3. Flow cytometric histogram representing the emitted fluorescence at FL1 (green- 530 nm) of an example of a non-ESBL producer isolate. (a) After treatment with CTX (4 mg/L) for 60 min; the MFI was 72. b) After treatment with CTX (4 mg/L) and CLA (4 mg/L) for 60 min: the MFI was 73.

predictive values of this test were 95.8 and 100%, respectively. Kappa testing showed an almost perfect agreement between the ESBL NDP test and disc diffusion method in detecting ESBLs (kappa =0.9).

Out of the 60 tested isolates, 42 (70%) and 18 (30%) were ESBL and non- ESBL producers, respectively with the flow cytometric assay method. The sensitivity and specificity of the test were 91.3 and 77.8% whereas the positive and negative predictive values of this test were 91.3 and 77.8%, respectively in comparison with the standard disc diffusion method (Table 1). The intrinsic autofluorescence signal of bacterial cells was detected at the first decade of the logarithmic scale [the mean fluorescence intensity (MFI) was 62]. This corresponds to very low fluorescence intensity without interference with the assessment of membrane depolarization using DIBAC4 (3) as a voltage sensor probe (Figure 2a). Higher intensity of green fluorescence (530/30 nm - FL1) was obtained with dead cells compared with viable cells; consequently, two distinct regions were defined, respectively, for depolarized and polarized cells after staining with DIBAC4 (3). Considering the ESBL-positive clinical isolates, the MFI was 293 after treatment with CTX for 60 min, and then drastically increased to 1541 following simultaneous incubation with both CTX and CLA for 60 min (Figure 2b, c). For non- ESBL producer isolates, the MFI was 72 after treatment with CTX (4 mg/L) for 60 min and remained around value 73 after treatment with both CTX and CLA for 60 min (Figure 3a,b).

For evaluation of agreement between the flow cytometric assay and disc diffusion method, Kappa testing showed substantial agreement between both tests (kappa= 0.7).

DISCUSSION

ESBLs are the main cause of resistance to beta-lactam antibiotics which are among the safest and most frequently prescribed antimicrobial agents all over the world. As their occurrence has been increasing, it becomes essential to evaluate their occurrence in *E. coli* and *K. pneumoniae* which are mostly ESBL producers (Pitout and Laupland, 2008; Sahu et al., 2011).

The incidence of ESBL-producing *K. pneumoniae* varies from country to another depending upon various factors, like antibiotic policy, the carriage rate among hospital personnel, and the type of disinfection used especially in the ICU (Sarojamma and Ramakrishna, 2011). It is recognized that Egypt has an extremely high rate of ESBL producers, with up to 70% of isolates producing the enzyme (Borg et al., 2006). In the present study, 76.6% (n= 46) of the 60 tested strains were ESBL producers and 23.3% (n=14) were non- ESBL producers. This could be attributed to the empirical usage of 3rd generation cephalosporins in treatment of nosocomial infections in our hospitals.

Although molecular methods brought speed and accuracy, they are coasty and not suitable for low income developing countries (Gazin et al., 2012). In this work, we assessed two phenotypic methods; the ESBL NDP test and flow cytometric assay for detection of ESBLs in *K. pneumoniae* clinical isolates in comparison with the standard disc diffusion method. The ESBL NDP test was able to detect all ESBL-producing isolates that hydrolyze

cefotaxime (color change from red to yellow in the first well), while the second well that contained tazobactam remained red (inhibition of hydrolysis), thus corresponding to a positive test. The sensitivity and positive predicative value of the test were 100 and 95.8%, respectively. This result was higher than that of Nordmann et al. (2012) who evaluated the ESBL NDP test retrospectively on a collection of 255 strains (from various clinical and geographical origins and previously characterized at the molecular level). In their published study, the sensitivity of the test was 92.6%. Also, our results are higher than those of Dortet et al. (2014) who applied the ESBL NDP test on 500 ESBL producing Enterobacteriaceae recovered from urine samples. They reported that the sensitivity of the ESBL NDP test was 98% while the positive predicative value was 98% which is higher than ours. The discrepancy of the results may be attributed to the different geographical origins and the large number of tested isolates in comparison with our study. Two false positive isolates were detected by the ESBL NDP test as some isolates could contain combined ESBL and AmpC-overproducing enzymes giving a positive result, if the corresponding AmpC hydrolyses cefotaxime at high level.

The specificity and the negative predictive value of the ESBL NDP test in our study were 85.7% and 100%, respectively. These results are lower than those of Nordmann et al. (2012) and Dortet et al. (2014) whereas, it was 100% in the first study and 99.8% in the second one. This could be explained by the inability of the test in detecting non-CTX-M ESBL producers and strains which had MIC values of cefotaxime lower that the resistance breakpoint for that molecule (>8 µg/ml).

Our results showan almost perfect agreement between the ESBL NDP test and disc diffusion method in detecting ESBLs (kappa =0.9) which agrees with those of Dortet et al. (2014) who observed a perfect correlation between cefotaxime resistance and positivity of the ESBL NDP test.

Compared to the standard disc diffusion method, flow cytometric assay yielded a sensitivity of (91.3%) while the specificity was 77.8%. It correctly detected 42 isolates out of the 46 ESBL positive isolates previously catalogued by the standard disc diffusion method. Only 4 strains tested false negative result which might be obtained whenever complex mutant or rare ESBL types are present as isolates expressing these enzymes confer resistance to cephalosporins but are partially inhibited or not inhibited by CLA acid, respectively (Canton et al., 2008; Drawz and Bonomo, 2010).

Our results are in concordance with those of Ramos et al. (2012) who tested 20 ESBL-negative and 41 ESBL-positive isolates phenotypically catalogued by the standard disc diffusion method and molecular typing. In their study flow cytometric analysis correctly detected all the 41 ESBL-positive isolates. It showed an excellent correlation either with phenotypic analysis or molecular typing however, in our study flow cytometric analysis showed substantial agreement with the standard disc diffusion method (kappa= 0.7).

The ESBL NDP test offers a simple and rapid test with an almost perfect agreement with the standard disc diffusion method in detecting ESBLs which could significantly help in guiding first-line antibiotic therapy and improve the outcome of infected patients. Flow cytometric assay is a promising method that might be used in the clinical microbiology laboratory provided that the availability of the device and a trained personnel. Nonetheless, the standard method remains the best one because of its low price for the lab and the patient.

Conflict of Interests

The authors have not declared any conflict of interest.

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Physicochemical and *in vitro* antimicrobial activity of the oils and soap of the seed and peel of *Citrus sinensis*

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Citrus sinensis **seed and peel oils were extracted by solvent extraction using n-hexane, after air drying and grinding. Soaps were formed by saponification methods. Fatty acid composition of the oil samples were analyzed using Gas Chromatograph-Flame Ionization Detector (GC-FID). Physicochemical properties of the oils and soaps were determined following standard methods. Antimicrobial activities were assessed by the agar disc and hole-in plate methods. The seed and peel oil yield were 38 and 30%, respectively and the colors were golden yellow and brownish-yellow, respectively. Physicochemical properties of the oil samples determined were: refractive index (RI): 1.46 and 1.47, smoke point: 140 and 149, flash point: 150 and 160, pH: 5.2 and 4.2, acid value (AV): 23.6 and 25.1 mgKOH/g, free fatty acid (FFA): 11.86% as oleic acid and 12.61% as oleic acid, iodine value (IV): 78.83I_2 g/100 g and 120.10I_2 g/100 g, peroxide value (PV): 18.00 mgKOH/g and 5.40 mgKOH/g, saponification value (SV): 222.58 and 41.25 mgKOH/g, ester value (EV): 178.24 and 28.96 mgKOH/g for the seed and the peel oil respectively. Inhibitory antimicrobial activities were assessed for the two oils and the soap produced at concentrations of 40 mg/ml and below, against most of the gram positive and gram negative bacteria as well as the two candida strains, screened as compared with streptomycin (1 mg/L) and acriflavin (6.3 mg/ml) standard controls. Seed oil demonstrated better activities than the peel oil with growth inhibitions obtained against *Staphylococcus aureus* and *Candida albicans* at a concentration as low as 2.5 mg/ml. This study has shown that the results obtained for the physicochemical and antimicrobial properties of the oils provide a synergy for the oil samples as suitable raw materials for the cosmetic and pharmaceutical industries**.

Key words: Physicochemical properties, antimicrobial activity, soap, seed oil, peel oil.

INTRODUCTION

Citrus sinensis (sweet orange) is one of the natural staple food of man, containing essential nutrients in adequate proportion. The nutritional and medicinal values of the fruit juice has made it essential and important part of

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human diet for ages (Okwu and Emenike, 2006; Ezejiofor et al., 2011). Generally, citrus are excellent sources of minerals, vitamins and enzymes. They have been reported to be free from fat and cholesterol, but contain important mineral elements such as potassium, calcium, phosphorus, magnesium and silicon (Assa et al., 2013). They are easily digested and bring about a cleansing effect on the blood and the digestive tract. Orange fruits have been discovered to have anti-scurvy property (Rapisararda et al., 1999). Furthermore, they are rich in vitamin C, folic acid and fiber; these contribute to the prevention of degenerative processes, particularly reducing the incidence and mortality rate of cancer as well as cardio- and cerebro-vascular diseases (Rapisararda et al., 1999; Cushnie et al., 2005; Pultrini et al., 2006).

C. sinensis belongs to the race *var. sinensis*, of the family Rutaceae. It is an hybrid between Pomelo (*C. maxima*) and Mandarin (*C. reticulata*) originating from Southeast Asia. The fruit size varies with cultivar and crop load, but most often measures between 2.5 to 4 .0 inches in diameter (Manthey, 2004). The shape of the fruit is spherical to oblong, with a peel thickness between that of grape fruit and tangerine, and is either smooth or roughly pebbly (Hilditch et al., 1950). It is usually very closely adhered to the flesh of the fruit. Its colour tints from green to light orange, depending on the cultivar. The presence and amount of seed depends also on cultivar, starting from 15 to 25 seeds per fruit (Nwobi et al., 2006). Of all the citrus fruits, *C. sinensis* is the commonest in the forest zone of Western Nigeria, Middle Belt, Eastern and some part of South-south Nigeria (Odbanjo and Sangodoyin, 2002).

The yield of orange juice is about half of the fruit weight thereby generating a very high amount of waste annually (Bovili, 1996). Citrus waste as huge as 36 metric tons are produced annually with Florida citrus industry generating 3.5 to 5 tons, used and sold as feed stock for cattle, and Nigeria generating about 0.3 million tons with potential to generate more annually (Ezejiofor et al., 2011). These agro wastes are common in Nigeria along major roads where retailers peel and sell to motorists and others. The wastes in market places constitute menace, causing environmental pollution.

Citrus fruit peels are also known to have flavonoids, an anti-oxidant (Bocco et al., 1998; Cushnie et al., 2005; Ghasemi et al., 2009). Essential oil had been generated in sweet orange and grape fruit (*C. paradisi*) peels (Ezejiofor et al., 2011; Okunowo et al., 2013) and the antimicrobial activities of grape peel oil had been documented (Okunowo et al., 2013). Essential oils in plant products have tremendous applications in food, cosmetic and aromatherapy (Ramadan et al., 1996; Haddouchi et al., 2013; Narmadha et al., 2013). Research in medicinal chemistry have also shown that screening plant products for antimicrobial activities have led to detection and development of new potential anti-infective agents (Ordonez et al., 2003; Arias et al., 2004; Rasool et al., 2008). The peel of citrus fruits is a rich source of flavones and many polymethoxylated flavones which are very rare in other plants (Ahmed et al., 2006). The antimicrobial abilities of essential oils from citrus plants have shown to be of particular interest for applications within the food industries (Caccioni et al., 1998).

In this study, the physicochemical properties and fatty acid compositions of the fixed oil from the seeds and peels of sweet orange were determined. Alkali generated from the peel and seed oil were used to prepare soaps. The antimicrobial properties of these oils and the soap were also determined with a view to investigate their suitability as possible alternative to the orthodox antibacterial soaps. The results of this investigation is expected to contribute to information on the usefulness of *C. sinensis* in the cosmetic industries for the health benefit of man and to reduce the menace of pollution caused by the peel wastes in the environment.

MATERIALS AND METHODS

Collection and preparation of sample

C. sinensis were collected mainly from Oje market in Ibadan, Oyo State, Nigeria (Specimen ID: 006653. Herbarium: PTBG). Its seeds and peel were manually removed and were then air dried to remove the moisture content. The dried seeds and peel were then grinded to particles with the aid of an electric grinding machine.

About 1430 and 2350 g of the ground *C. sinensis* seed and peel were weighed separately and were transferred into a porous thimble and kept in the Soxhlet apparatus for extraction. Anti-bumping granule was dropped into the flask to prevent the build of pressure in the flask and n-hexane was added as the extracting solvent. The oil was recovered from the mixture by evaporating the residual extracting solvent using a rotary evaporator. The weight of oil was noted (Soxhlet, 1879, Laurence et al., 2012).

After the extraction, the oil was transferred into a weighed round bottom flask. The weight of the oil was determined by weighing the oil and the flask and subtracting the weight of empty flask. The percentage yield was determined.

Physical properties of the oils

The specific gravity of the seed and peel oil were determined by measuring 10 mL of the oil samples into a pre-weighed measuring cylinder. The values obtained were used to determine the specific density of the oil. The pH of the oils were determined using Hannah instruments, pH 210 Microprocessor pH meter while the refractive index was determined at room temperature using the Abbey refractometer at the Department of Pharmaceutical Chemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.

Other physical parameters such as flash and smoke points, cloud and pour points and viscosity test were carried out using ASTM D56 (2001), TCWI (2009) and ASTM D445 (1965).

Chemical properties of the oils

The chemical properties such as the acid value (AV), free fatty acid (FFA), Iodine value (IV), saponification value (SV) and peroxide value

(PV) of the seed and peel oil were determined by standard method of AOAC (1990).

Determination of fatty acid composition

Fatty Acid composition of the oil samples were analyzed using PERKIN Elmer Clarus 500 Gas Chromatograph employing the following conditions: capillary column (RT-2560, 50 m x 0.25 mm ID, 0.25 micron dry film); Nitrogen was used as a carrier gas, a flame ionization detector and a sample volume of 1.0 L was employed. The temperature programming of the instrument: Initial temperature was 50°C held for 5 min, with an increase of 4/min to 190, then 0.8/min to 212, then 0.4°C /min to 220. The total GC-FID running time was 85.49 and 78.90 min for the orange peel oil and orange seed oil respectively.

Preparation of orange peel ash

The oranges were peeled and the peels were washed with double distilled water and dried in an oven at (105°C ± 2) for two days to constant weight. The dried peels were ashed in a porcelain crucible placed in a Gallenkamp muffle furnace for 6 h by stepwise increase of the temperature up to 500°C. The ashed samples were homogenized in porcelain mortar and pestle and sieved. Sixty (60) g of the sample were weighed into poly ethylene buckets of 2 L capacities and one liter of water was added (Onyegbado et al., 2002; Olabanji et al., 2012). The buckets were covered to prevent contamination and extractions were done for 24 h. The extracts were carefully decanted and double distilled water were added in ratios of 1:4 of sample to double distilled water and were analyzed by atomic absorption spectrophotometer (AAS) Buck Model 205 at the Center for Energy Research Development, Obafemi Awolowo University, Ile- Ife. These extracts were alkaline to litmus paper and methyl orange.

Determination of molarity of orange peel ash alkali

Primary standard (Na_2CO_3) of known molarity was prepared and used to standardize the acid (HCl) which was titrated against the derived alkali using methyl orange indicator to determine its molarity.

Saponification reaction using the ash-extracts

Two hundred milliliter of the ashed peel extract was concentrated to 50% by heating in a beaker (Babayemi et al., 2011); excess of alkali is usually recommended in order to ensure complete saponification of the oil/fat and to retain the antibacterial effect of the alkalis (Kirk et al., 1954). The concentrated extract was heated to 60 to 70°C and 15 g of oil was gradually charged into the pot. The temperature was maintained at 70°C and 5 ml of double distilled water was added intermittently with continuous stirring until the mixture was semi solid and creamy in color, 10 ml of brine was charged into the beaker content and the soap was homogenized. The soap was scooped from the upper layer when the content of the beaker had cooled and the lye discarded. The soap was washed by pouring water on it.

Analysis of soap produced

Determination of total fatty matter (TFM)

The TFM was determined by the petroleum spirit extraction method. Soap (1 g) was dissolved in 10 ml of warm water and transferred to a separating funnel. Two drops of methyl orange indicator were added, followed by 4N H_2SO_4 until the indicator color changed from orange to pink. Petroleum spirit 1mL was added and the separating funnel shaken vigorously for 30 s. The solution was then allowed to settle for a few minutes until the fatty acid liberated from the soap formed a clear layer on top. The soap was skimmed off, washed with distilled water and dried to constant weight in an oven at 60°C. The percent total fatty matter was determined from the weight obtained for the fat and the soap.

Determination of total alkali

The total alkali was determined by titrating excess acid contained in the aqueous phase with standard volumetric NaOH solution. Five millilitre of ethanol was added to 1g of finished soap after which 0.5 ml of 1N H_2SO_4 solution was added to the mixture and heated till the soap sample dissolved. Test solution was titrated against 1 N NaOH using phenolphthalein as indicator. The total alkali was obtained following AOAC (1990).

Foamability test

About 0.5 g of the soap was added to a 100 ml standard flask containing 100 ml of double distilled water. The mixture was shaken vigorously two minutes to generate foams. The flask was allowed to stand for 10 min. The height of the foam in the solution was noted.

Antimicrobial activity assays

Test organisms

Microorganisms used include reference and clinical isolates comprising of Gram positive and Gram negative bacteria and fungi strains. These include *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* (clinical strains), *Shigella flexinerii* (clinical strain), *Klebsiella pneumonia* (clinical strain), *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* NCIB 3610. *Candida albicans* ATCC 24433 and *Candida pseudotropicalis* NCYC 6 were the fungi strains used. The strains were from stocks of culture collections maintained in the Pharmaceutical Microbiology Laboratory of the Department of Pharmaceutics, Faculty of Pharmacy, Obafemi Awolowo University where the experiments were performed.

Agar diffusion tests: Disc diffusion and cup plate methods

The disc diffusion test was used for the pure oils and the soaps while the cup plate test was used for their dilutions. The oil samples and their soap preparations were dissolved in MeOH/H_2O to give varying concentration of 2.5, 5.0, 10.0, 20.0 and 40.0 mg/ml.

Surface plating of the organisms were done for the 20 ml oven dried Mueller Hinton Agar used for the overnight grown bacteria and the Sabouraud Dextrose Agar used for the fungi strains. For the dilutions, holes of diameter 9 mm were made in the agar plates using a sterile metal cup-borer. Two drops of each dilution and control were put in each hole under aseptic condition, kept at room temperature for 1 h to allow the agents to diffuse into the agar medium and incubated accordingly. For the pure oil and soap, each of these were used to soak sterile 6 mm Whatman paper discs and subsequently placed on the agar plates, allowed for diffusion and incubated. Streptomycin (1mg/ml) and acriflavine (6.3 mg/ml) were used as positive controls for bacteria and fungi respectively. MeOH/H_2O and Tween 80 were the negative controls. The plates were incubated at 37°C for 24 h for the bacterial strains and at 25°C

Figure 1. Seed oil and peel oil.

for 72 h for the fungal strains. Antimicrobial activity was evaluated by noting the zone of inhibition against the test organisms.

RESULTS AND DISCUSSION

The percent yield of the oils was 38 and 30% for seed oil and peel oil, respectively. Using same extraction process but different solvent Nwobi et al. (2006) got 36% yield for the orange seed oil, this is close to the value in this study.

The oil of orange seed and peel showed acidic pH values (4.2 and 5.2). These values were however higher than 3.69 reported by Nwobi et al. (2006) for the peel oil. The pH values indicated that the seed oil is more acidic than the peel oil probably due to presence of more fatty acids in the seed oil.

The seed oil has a golden-yellowish color (Figure 1); similar result was obtained by Nwobi et al. (2006) while the peel oil has a brownish-yellow color, similar to the yellow color obtained in Okunowo et al. (2013) grape peels oil. The yellow color may be an indication of carotenoids, a fat, soluble in humans due to the presence of long unsaturated aliphatic chains as in some fatty acids. Carotenoids are known as provitamin A. They act as precursors to the production of vitamin A in the body which performs several biological functions within the body. They also act as antioxidants (Sommer and Vyas, 2012).

Refractive Index decreased with unsaturation and molecular weight of the fatty acids. The refractive index of the seed oil and peel oil was 1.46 and 1.47, respectively. This corroborates the findings of Nwobi et al. (2006) and Ezejiofo et al. (2011). This indicates that the seed oil, compared with the peel oil, has a lower unsaturation and a lower molecular weight of fatty acids. The lower molecular weight of fatty acid is suggestive of its higher saponification value since saponification value is inversely proportional to the mean molecular weight of fatty acids (Dimberu et al., 2011). The smoke point, flash point and free fatty acid content of the oils have a linear relationship. The higher the free fatty acid content of an oil, the lower the smoke point. The smoke point and flash point of the seed oil were 140 and 150°C, respectively while that of the peel oil were 149 and 160°C, respectively. Nwobi et al. (2006) obtained 149°C in orange seed oil which is still within the values found in seed and peel oils of this study.

The seed oil has a specific density of 0.997 g/cm^3 while the peel oil has a value of 0.788 g/cm^3. The value obtained from Ezejiofor et al. (2011) study lies within the range of density of the oils in this study. Density of seed oils depends on their fatty acid composition, minor components and temperature (Table 1).

Acid value accounted for the presence of free fatty acids in the oils as an indicator of the presence and extent of hydrolysis of lipolytic enzymes and oxidation and it is used as an indicator of edibility of an oil. The values indicated that the oils were non edible because it was above the limit of 10 mg KOH/g of oil and found to be unsuitable for dietary purposes (Barkatullah et al., 2012) and 0.6 mg KOH/g FAO/WHO (1993), as the peel oil contain higher fatty acid contents.

The free fatty acid content in seed oil which is 11.86% (as oleic acid) is lower than that of peel oil which is 12.61% (as oleic acid). This indicates that the oils could readily react with metal salt to generate soaps since the FFA were far above the 2.5 and 1.376% FAO/WHO recommended for coconut and palm oil respectively (FAO/WHO, 1993). High FFA nullified their edibility.

Peroxide value serves as a common indicator of lipid oxidation. Orange seed oil has a peroxide value of 18.00 millieq/kg while the peel oil has a value of 5.40 millieq/kg. This indicates that the seed oil has undergone primary oxidation than the peel oil since peroxide value gives a measure of the extent to which an oil sample has undergone primary oxidation. The peroxide value of the peel oil is within the acceptable range of 10.00millieq/kg FAO/WHO (FAO/WHO, 1993) while that of seed oil was above indicating that lipid oxidation had occurred.

The saponification value of the seed oil is found to be 222.58 mg KOH/g while it is 41.25 mg KOH/g for the peel oil. The higher saponification value of the seed oil shows the presence of lower molecular weight fatty acids in the oil and it may therefore be regarded as more edible than the peel oil.

The Iodine value of the seed oil is 78.00 gI_2/100 g which is lower than that of the orange peel oil which is 120.10 gI_2/100 g indicating that orange peel oil is rich in unsaturated fatty acid (70.05%). This implies that orange seed oil has a lower amount of double bond (59.76% unsaturated fatty acid) thus lowering the susceptibility of such oil to oxidative rancidity. Triglyceride oils are divided into three groups depending on their iodine values: drying, semi-drying and non-drying oils. The iodine value of a drying oil is higher than 130. This value is between90 and 130 for semi-drying oils. If the iodine value is smaller than 90, oil is called non-drying oil (Guner et al., 2006).

This classifies orange seed oil as a non-drying oil and

Table 1. Physico-chemical parameters of the oil samples.

Variable	Orange seed oil	Orange peel oil
pH	4.2	5.2
Colour	Golden-yellowish	Brownish -yellow
Percentage yield	38%	30%
Specific density	0.997 g/cm^3	0.778 g/cm^3
Refractive Index	1.46	1.47
Smoke point	140°C	149°C
Flash point	150°C	160°C
Cloud point	13°C	16°C
Pour point	7°C	10 °C
Viscosity 100°C	3.8185cst	0.9622cst
Viscosity 40°C	11.968cst	1.9766cst
Acid value	23.6 mgKOH/g	25.1 mgKOH/g
Peroxide value	18.00 mgKOH/g	5.40 mgKOH/g
Free fatty acid	11.86% as oleic acid	12.61% as oleic acid
Saponification value	222.58 mgKOH/g	41.25 mgKOH/g
Ester value	178.24 mgKOH/g	28.96 mgKOH/g
Iodine value	78.83 I_2g/100 g	120.10 I_2g/100 g

Table 2. Fatty acid composition of the seed oil.

Saturated fatty acid (relative abundance, %)	Monounsaturated fatty acid (relative abundance, %)	Polyunsaturated fatty acid (relative abundance, %)
Palmitic acid C16:0 (31.1)	Palmitoleic acid C16:1 (0.34)	Linoleic acid C18:2n6c (35.13)
Stearic acid C18:0 (4.97)	Oleic acid C18:1n9c (24.95)	Dihomo-linolenic acid C20:3n6 (0.04)
Arachidic acid C20:0 (3.68)	-	-
Heneicosylic acid C21:0 (0.32)	-	-
Tricosylic acid C23:0 (0.16)	-	-
Total = 40.23	Total = 25.29	Total = 35.17

the peel oil as a semi-drying oil. The peel oil will be more applicable in varnishes and paint industry while the seed oil will be useful in soap industry. The seed oil contains 59.76% unsaturated fatty acid, peel oil has 70.05% unsaturated fatty acid, 24.59% mono-unsaturated fatty acid and 35.17% polyunsaturated fatty acid (Table 2) while the peel oil has 70.05% unsaturated fatty acid, 31.83% mono-unsaturated fatty acid and 38.22% polyunsaturated fatty acid (Table 3). This implies that the peel oil is more unsaturated than the seed oil thereby confirming the reason for its higher iodine value, smoke point and higher refractive index. This also predicts the more oxidation stability of the seed oil and its possibility of serving as edible oil.

From the fatty acid profiles represented in Tables 2 and 3), it indicates that the peel oil has a high proportion of fatty acids with high molecular weight and this explains its low saponification value (Table 1). This is because they have relatively fewer numbers of carboxylic functional groups per unit mass of the oil. Thus it is regarded non-edible and may not be suitable for soap making. The higher percentage of unsaturation (mono and polyunsaturation) in peel oil makes it more reactive and useful in industrial application such as surface coating applications for example, paints, vanishes, printing and writing inks. The seed oil contains one out of the two families of essential fatty acid which is linoleic acid (omega-6) and it is the most abundant unsaturated fatty acid with a relative abundance of 35.13%. The peel oil contains the two families of essential fatty acid which is linoleic acid (omega-6) 18.63% and -linoleic acid 3.62%. Palmitoleic acid (omega-7) is the most abundant unsaturated fatty acid with a relative abundance of 22.78% in the peel oil.

The peroxide value of the orange seed oil exceeds the permitted maximum peroxide value for edible oil, which is 10 mequivalent of oxygen/kg of the oil (FAO/WHO, 1993) and its high acid value, coupled with high percentage of saturated fatty acid indicate that the orange seed oil may not be good for consumption but useful in industrial applications such as the cosmetics industry which includes soap making, perfumes and unguents.

The metal analysis (Table 4) of the peel ash showed metals of varying concentrations. Although the soap produced from the ash-derived alkalis was softer than bar

Table 3. Fatty acid composition of peel oil.

Saturated fatty acid	Relative abundance %	Monounsaturated fatty acid	Relative abundance %	Polyunsaturated fatty acid	Relative abundance %
Undecylic acid C11:0	10.83	Palmitoleic acid C16:1	22.78	Linoleic acid C18:2n6c	18.63
Lauric acid C12:0	0.88	Oleic acid C18:1n9c	9.05	-Linolenic acid	3.62
Palmitic acid C16:0	0.75	-	-	-	-
Stearic acid C18:0	2.61	-	-	Dihomo-linolenic acid C20:3n6	7.46
Eicosanoic acid C20:0	1.69	-	-	Arachidonic acid C20:4n6	1.63
Behenic acid C22:0	0.48	-	-	Cis-13,16- docosadienoic acid C22:2	6.88
Tricosylic acid C23:0	8.41	-	-	-	-
Lignoceric acid C24:0	4.32	-	-	-	-
Total	29.97	-	31.83	-	38.22

Table 4. Concentrations and percentage compositions of ash derived alkali from peels.

Elements	Concentration (ppm)	Composition of elements (%)
K	151.97	68.77
Ca	39.7	17.96
Na	24.7	11.17
Mg	4.62	2.08
Total	220.97	

Figure 2. Soap from ashed peel alkali and seed oil.

Table 5. Physicochemical analysis of the soap produced.

Colour of soap	Yellow
pH	9.79
Total fatty matter	41.0%
Total alkali	4.65%
Foam height	7 cm^3
Solubility in water	Soluble
Texture	Soft

Figure 3. Soap foamability test.

soap in the market it could still be described as soft solid soap (Figure 2). This is expected as the percentage concentrations of K, Ca, Na, Mg in the peel were 68.77, 39.7, 24.7 and 4.62% respectively (Table 4) of the total metal ions analyzed in the sample. The solubility of soap in water increased with the size of the monovalent cation (base); an increase in the size of a divalent cation (Mg, Ca) results in a decrease in the foamability. Potassium soaps are more soluble in water than sodium soaps; hence, the soap produced was soluble and lather very well (Figure 3, Table 5). Potassium soaps in concentrated form are called soft/liquid soap. Potassium soaps require less water to liquefy because of their softness and greater solubility; thus can contain more cleaning agent than liquefied sodium soap and can be used as shampoos, shaving creams, cleaning of dirty floors and cooking utensils, in emulsion polymerization processes used in rubber and plastic industries and in such other

Table 6. *In-vitro* antimicrobial activity of the oil and soap of the seed and peel of *C. cinensis.*

Agent	Organisms	Concentration (mg/ml)	Diameter of zone of inhibition (mm)**
Peel oil	*P. mirabilis* (clinical strain)	Pure oil	16.0
	K. pneumonia (clinical)	Pure oil	2.0
	P. fluorescence (clinical strain)	40	4.0
	S. aureus (ATCC 29213)	20	6.0
		40	8.0
	Shigella flexinerii (clinical)	40	5.0
	C. albicans (ATCC 24433)	20	4.0
		40	7.0
	C. pseudotropicalis (NCYC 6)	40	10.0
Seed oil	*E. coli* ATCC 25922	Pure oil	6.0
		40	2.0
	B. subtilis (NCIB 3610)	Pure oil	3.0
		20	2.0
		40	8.0
	Proteus mirabilis (clinical strain)	Pure oil	14.0
	K. pneumonia (clinical strain)	Pure oil	5.0
		40	4.0
	Ps. aeruginosa ATCC 27853	10	2.0
		20	3.0
		40	5.0
	Ps. fluorescence (clinical strain)	40	4.0
	S. aureus (ATCC 29213)	2.5-5.0	6.0
		10	7.0
		20	8.0
		40	14.0
	Shigella flexineri (clinical strain)	20	4.0
		40	11.0
	C. albicans (ATCC 24433)	2.5	2.0
		5.0-10.0	4.0
		20	6.0
		40	10.0
	C. pseudotropicalis (NCYC 6)	10	4.0
		20-40	10.0
Seed oil soap	*Proteus mirabilis* (clinical strain)	Pure Soap	4.0
	Klebsiella pneumonia (clinical strain)	Pure Soap	9.0

Diameter of zone of inhibition of streptomycin (1 mg/ml) for each organism was: *E. coli* ATCC 25922, 14.0 mm; *P. aeruginosa* ATCC 27853, 14.0 mm; *P. fluorescence* (clinical strain), 14.0 mm; *S. aureus* ATCC 29213, 14.0 mm; *B. subtilis* NCIB 3610, 10.0 mm; *K. pneumonia* (clinical), 12.0 mm; *S. flexinerii* (clinical) 10.0 mm; *P. mirabilis* (clinical strain), 10.0 mm. Acriflavin (6.3 mg/ml) inhibition for the fungi was: *C. albicans* (ATCC 24433),18.0 mm, *C. pseudotropicalis* (NCYC 6), 21.0 mm. *The agents showed activities only against the organisms indicated. **Zone of inhibition less cup size.

similar uses. The presence of 24.7% sodium out of the total percent of the alkali increases the firmness of the soap which ought to be liquid or semi-solid. Calcium is the major ion that limits its foam ability because of 39.7% composition.

The yellowness of the oil was considerably reduced by bleaching, which gave the soap a cream colour. Spectrophotometry analysis of the metallic ions present in ashed samples solution (Table 4) showed that the alkali consist of ions that are essential diet components by contributing sodium, calcium, potassium and other essential nutritional elements.

Results of antimicrobial evaluation show that the two oil samples possess useful antimicrobial activities as anti-bacterial and antifungal inhibitory activities were obtained at concentrations of 40 mg/ml and below (Table 6). The

antimicrobial activities of grape fruit (*C. paradisi*) and grape peel oil had earlier been documented (Okunowo et al., 2013). Furthermore, the presence of metabolites with documented antimicrobial effects such as alkaloids, saponins, flavonoids, tannins and phenolic compounds in *C. sinensis* peel extract has been reported (Bocco et al., 1998; Hussain et al., 2015). Thus the antimicrobial activities obtained in this study have known scientific basis. The antimicrobial activities are broad spectrum against a wide range of Gram positive and Gram negative bacteria and the two candida strains, *C. albicans* and *C. pseudotropicalis*, screened. These organisms have been implicated in skin and mucous membrane infections with reports of morbidity and mortality (Mahmoud, 2001). Seed oil demonstrated better activities than the peel oil indicating that antimicrobial constituents are more concentrated in the seed oil.

Further studies are therefore needed to elucidate these constituents and their contributions to the antimicrobial effects. These results also indicate that free fatty acids, obtained at a higher content in the peel oil compared with the seed oil, do not contribute to the antimicrobial effects of *C. sinensis*. Inhibition zones were obtained for the seed oil against *S. aureus* and *C. albicans* at a concentration as low as 2.5 mg/ml. In some cases the activities of these oils were observed to be comparable to that obtained for the standard antibacterial agent, streptomycin, at the tested concentration. These cases include inhibitory activities obtained for the pure peel and seed oil against *Proteus mirabilis* (16 and 14 mm, respectively) compared with that for streptomycin [1 mg/ml] which was 10 mm.

Seed oil at 40 mg/ml also demonstrated similar inhibitory activity with streptomycin at 14 mm zone of inhibition. The activity of the seed oil against *P. aeruginosa* at a concentration as low as 10 mg/ml is especially noteworthy as this organism is notorious for its intrinsic resistance to most standard antibacterial agents. For the soap, antimicrobial activities were obtained only for the seed oil soap with activities demonstrated against *P. mirabilis* and *K. pneumonia* at 4.0 and 9.0 mm zone of inhibition respectively.

The antimicrobial activities of the pure seed oil and peel oil showed its usefulness in cosmetic and pharmaceutical industries in preparation of topical cream/gel against both gram -positive and gram negative bacteria and fungi infection. The activities of the seed oil soap further strengthen the usefulness of the seed oil for potential use in soap formulation against susceptible organisms. The peel oil will also find good use as antimicrobial agent in many infectious diseases especially against infections caused by *S. aureus*. It also has great potential as antifungal agents against the candida strains (Table 6).

Conclusions

From the physicochemical parameters and fatty acid composition of *C. sinensis* seed and peel oil analyzed, both oils are recommended for industrial applications, specifically the cosmetics industry. High composition of unsaturated fatty acid such as palmitoleic acid, linoleic acid, cis-13, 16- docosadienoic acid, alpha-linoleic acid and arachidonic acid in the peel oil makes it reactive and to have a semi-drying property as confirmed by its iodine value. Thereby making it suitable in the production of paints, inks and vanishes.

The presence of fatty acids such as linoleic acid, palmitoleic acid, oleic acid and other unsaturated fatty acid in the seed oil could function as emollient and thickening agents. They also serve as fragrance ingredient and cleansing agents. Linoleic acid is an anti-oxidant which could prevent ageing. Saturated fatty acids such as palmitic acid, stearic acid and arachidic acid fulfill the role of a fragrance ingredient, thickener or hardener when the oil is used in soap making.

The broad spectrum activities of the seed oil against strains of organisms responsible for many infectious diseases together with the favourable physicochemical properties obtained for this oil, which support its use in cosmetic and soap making, are actually synergistic and make this oil of tremendous potential for these industries. This study has shown that *C. sinensis* seeds and peels could be put to productive use in the cosmetic and pharmaceutical industries rather than continuing to constitute worrisome menace as environmental wastes and pollutants.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

The growth potential and antimicrobial susceptibility patterns of *Salmonella* species and *Staphylococcus aureus* isolated from mobile phones of food handlers and health care workers in Jimma Town, Southwest Ethiopia

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Mobile phones are increasingly being used by all people in day to day life. However, they are found suitable breeding grounds for various pathogenic microorganisms. This study was aimed to determine the growth potential and antimicrobial susceptibility patterns of *Salmonella* species and *Staphylococcus aureus* isolated from mobile phones of food handlers and health care workers in Jimma Town, Southwest Ethiopia. Collection of mobile phone cotton swab samples and laboratory based microbiological analysis was used as the study design. A total of 188 mobile phones were sampled from food handlers and health care workers. The growth potential of *Salmonella* spp. and *S. aureus* isolated from mobile phones was assessed in various food items. The results have shown that *Salmonella* spp. and *S. aureus* isolated from mobile phones of food handlers and health care workers were found growing to their infective dose within 12 to 18 h in the sampled food items. Regarding the antimicrobial susceptibility test patterns, *Salmonella* spp. isolates were susceptible to ciprofloxacin, norfloxacin, gentamycin, chloramphenicol, and kanamycin, though they were highly resistant to ampicillin and nalidixic acid. On the other hand, *S. aureus* isolates were susceptible to gentamycin, chloramphenicol, amikacin, ciprofloxacin, streptomycin, and kanamycin. In multidrug resistance patterns, 5 and 6 drugs resistance were observed in *Salmonella* spp. and *S. aureus,* respectively. This indicates that mobile phones could play a significant role in spreading drug resistant infectious agents within the community. Therefore, the outmost care should be taken in using mobile phones.

Key words: Growth potential, microbial pathogens, mobile phones.

INTRODUCTION

Mobile phones are increasingly being used by all people in day to day life. They become in contact with various surfaces and are thus likely to be getting contaminated with various organisms (Tambe et al., 2012). Mobile phones make most human activities easier; however, they pose a number of serious public health problems as well (Czapiński and Panek, 2011).

Several pathogenic microbes including *Salmonella* species and *Staphylococcus aureus* have been isolated in different countries from mobile phones by many

researchers (Ekrakene and Igeleke, 2007; Akinyemi et al., 2009; Al-Abdalall, 2010). The presence of pathogenic microbes on mobile phones could indicate unknowingly that the devices had played a great role in spreading the infectious agents within the community and cause disease outbreaks (Akinyemi et al., 2009). The subscription of mobile phone technology is highly increased in today's world. It is estimated that in Ethiopia, approximately 40 million people have their own mobile phones, including adults and children. However, to the knowledge level of the investigator, there has been no published data on the growth potential of microbes isolated from mobile phones. Therefore, this study was aimed to determine the growth potential and antimicrobial susceptibility patterns of *Salmonella* spp. and *S. aureus* isolated from mobile phones of food handlers and health care workers in Jimma Town, Southwest Ethiopia.

METHODOLOGY

The study site and period

The study was conducted in Jimma town which is located at 353 km Southwest of Addis Ababa, Ethiopia. The geographical coordinates of the town are 7°41'N latitude, 36°50'E longitude (Abebe et al., 2011). The study was conducted from September 2012 to June 2013.

Study design and population

Collection of mobile phone swab samples and laboratory based microbiological analysis was used as the study design. A total of 188 mobile phone user samples including 119 health care workers and 69 food handlers were taken as the study population. The selection of study population participants was based on using purposive sampling technique. The sample size was determined using the statistical formula developed by Kothari (2004).

Sample collection

The sampled mobile phones were aseptically swabbed using sterile cotton moistened with normal saline solution by rolling it over exposed outer surface of the mobile phones. The cotton swabs were placed into a tubes containing 10 ml sterile normal saline and kept in ice box and transported to Research and Postgraduate Laboratory, Department of Biology, College of Natural Sciences, Jimma University for microbiological analysis. The microbiological analysis was done after two-three hours of sample collection following standard microbiological methods.

Inoculation and enumeration

Isolation of S. aureus

One milliliter of each mobile phone swab samples was transferred aseptically into 9 ml of buffered peptone water (BPW) and vortex mixed thoroughly for 5 min. The homogenates were serially diluted from 10^{-1} to 10^{-6} and a volume of 0.1 ml aliquot of appropriate dilution was spread-plated on pre-solidified plates of Mannitol salt agar (MSA). The plates were incubated at 37°C for 24 h.

Identification of S. aureus

Golden yellow colonies from the MSA plates were aseptically picked and transferred into 5 ml nutrient broth and incubated at 37°C for 24 h for further purification. Then, a loopful of culture from the nutrient broth was streaked on nutrient agar supplemented with 0.75% NaCl and again incubated at 37°C for 24 h. Finally, the distinct colonies were characterized using the established microbiological methods (Acco et al., 2003). Gram-positive cocci with cluster arrangement under the microscope were subjected to preliminary biochemical tests (coagulase, catalase, and oxidase).

Isolation of Salmonella spp.

To test the presence of *Salmonella* spp. in the sampled mobile phones, 1 ml cotton swab sample of each mobile phone was aseptically transferred into a tube containing 9 ml of buffered peptone water, homogenized for 5 min and then incubated at 37°C for 24 h for recovery of the organism (Primary enrichment). Following the buffered peptone water primary enrichment, 1 ml of the culture from the buffered peptone water was transferred into 10 ml of selenite cysteine broth (Oxoid) and was incubated at 43°C for 48 h (Secondary enrichment) (Johnson and Case, 2007).

Identification of Salmonella spp.

A loopful of suspension from selenite cysteine broth (Oxoid) tube was streaked onto Xylose Lysine Deoxycholate (XLD) agar plate (Oxoid) and incubated at 37°C for 18 h.

Characteristic black centered red colonies from the selective media were picked, further purified and biochemically tested (Triple iron sugar agar, Lysine iron agar, Simmons Citrate agar, Urea agar and SIM media) based on standard methods (Johnson and Case, 2007).

Determination of the growth potential

In order to standardize the procedure (*Salmonella typhi* ATCC13311 and *S. aureus* ATCC25923) were used as a control in this study. The growth potential of *Salmonella* spp. and *S. aureus* isolated from mobile phones was assessed in food item (Injera and Bread). 200 g of each food item was steamed at 80°C for 10 min to kill any vegetative cell, including *Salmonella* spp. and *S. aureus* which might be present in the items. Steamed food (10 g each) was examined for aerobic mesophilic bacteria and aerobic bacterial spores. Then, 100 g of each street food item was challenged separately with 1 ml overnight culture of the test strains to give an inoculums level of 10^2 to 10^3 cfu/g. To determine the initial inoculums level, 10 g of each freshly inoculated food was homogenized in 90 ml of BPW and 0.1 ml of appropriate dilution

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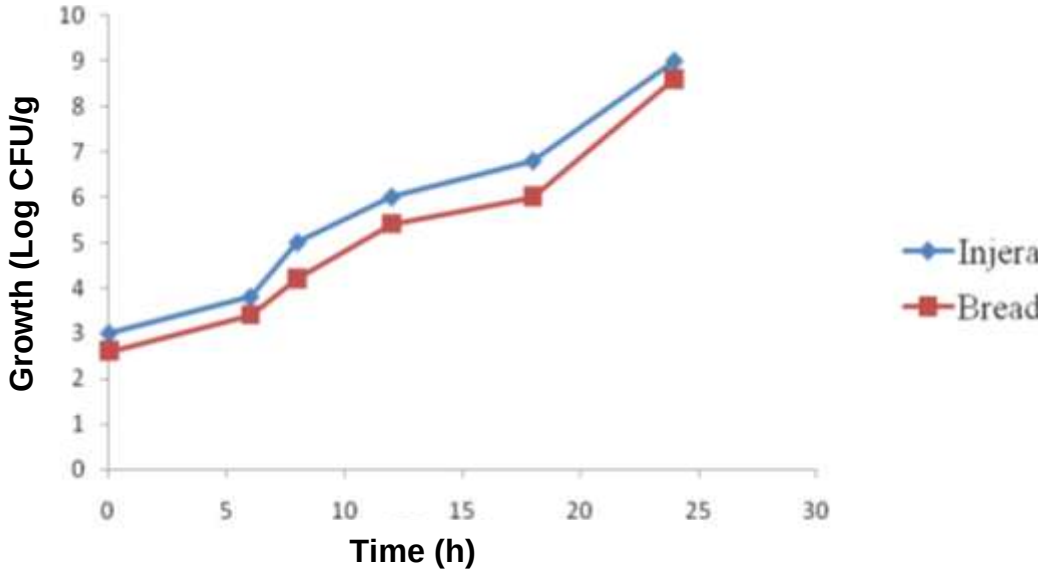

Figure 1. The growth potential of *S. aureus* isolated from mobile phones, Jimma town, Southwest Ethiopia.

was spread plated on XLD for *Salmonella* spp. and MSA for *S. aureus* agar plates to count *Salmonella* spp. and *S. aureus.* A portion of food sample was further sampled aseptically at 6 h interval from 0 to 24 h (Muleta and Ashenafi, 2001).

Antimicrobial susceptibility test for *Salmonella* spp. and *S. aureus* isolates

Antimicrobial susceptibility test for *Salmonella* spp. and *S. aureus* isolated from mobile phones was performed using the disk diffusion method in Mueller Hinton Agar (Oxoid). Briefly, a standardized suspension of the bacterial isolates was prepared and the turbidity of the inoculums was matched with the turbidity standard of 0.5 McFarland (Bauer et al., 1966). The results were interpreted as per the criteria of the National Committee for Clinical Laboratory Standards Institute (Wikler et al., 2007). The isolates were categorized into resistance, intermediate, and susceptible based on their zone diameter measurements. The intermediates were considered as resistant in this study. Drug disk with their defined concentration, chloramphenicol (30 μgml^{-1}), ciprofloxacin (5 μgml^{-1}), clindamycin (2 μgml^{-1}), erythromycin (15 μgml^{-1}), gentamycin (10 μgml^{-1}), kanamycin (30 μgml^{-1}), penicillin (10 μgml^{-1}), amikacin (30 μgml^{-1}), streptomycin (10 μgml^{-1}), and tetracycline (30 μgml^{-1}) were used for *S. aureus* and ampicillin (10 μgml^{-1}), nalidixic acid (30 μgml^{-1}), kanamycin (30 μgml^{-1}), tetracycline (30 μgml^{-1}), chloramphenicol (30 μgml^{-1}), norfloxacin (10 μgml^{-1}), gentamycin (10 μgml^{-1}), ciprofloxacin (5 μgml^{-1}), and streptomycin (10 μgml^{-1}) were used for *salmonella* spp.

RESULTS

From a total of 188 mobile phone samples examined for microbiological safety, 41.5% of them were found positive for *S. aureus*. Over 22.5% of them were isolated from mobile phones of food handlers, whereas 19% were from health care workers mobile phones. On the other hand, 11.70% of the sampled mobile phones were positive for *Salmonella* spp. Specifically, *Salmonella* spp. was isolated from 6.38% of health care workers and 5.32% of food handler mobile phones.

In challenge studies, *S. aureus* isolates had increased by 1.5 Log CFU/g within 8 h in both food items (Bread and Injera). The growth rate in the first 8 h had shown steady increase and then finally reached counts of ≥9 Log CFU/g at 24 h (Figure 1).

Salmonella spp. isolates reached counts of ≥8 Log CFU/g within 24 h in both food items (Bread and Injera). There was about 1.2 Log CFU/g increase in the first 6 h and then a steady growth has been found thereafter. Relatively lower growth rate was observed in bread than in the injera (Figure 2).

Antimicrobial susceptibility test

Salmonella spp. isolates were susceptible to ciprofloxacin, norfloxacin, gentamycin, chloramphenicol, and kanamycin; though they were highly resistant to ampicillin and nalidixic acid (Table 1). In multidrug resistance pattern, 5 drugs resistance were observed in *Salmonella* spp. isolates.

S. aureus isolates were susceptible to gentamycin, chloramphenicol, amikacin, ciprofloxacin, streptomycin, and kanamycin. However, the isolates were found highly resistant to penicillin G and clindamycin (Table 2). In multidrug resistance pattern, 6 drugs resistance were observed in *S. aureus* isolates.

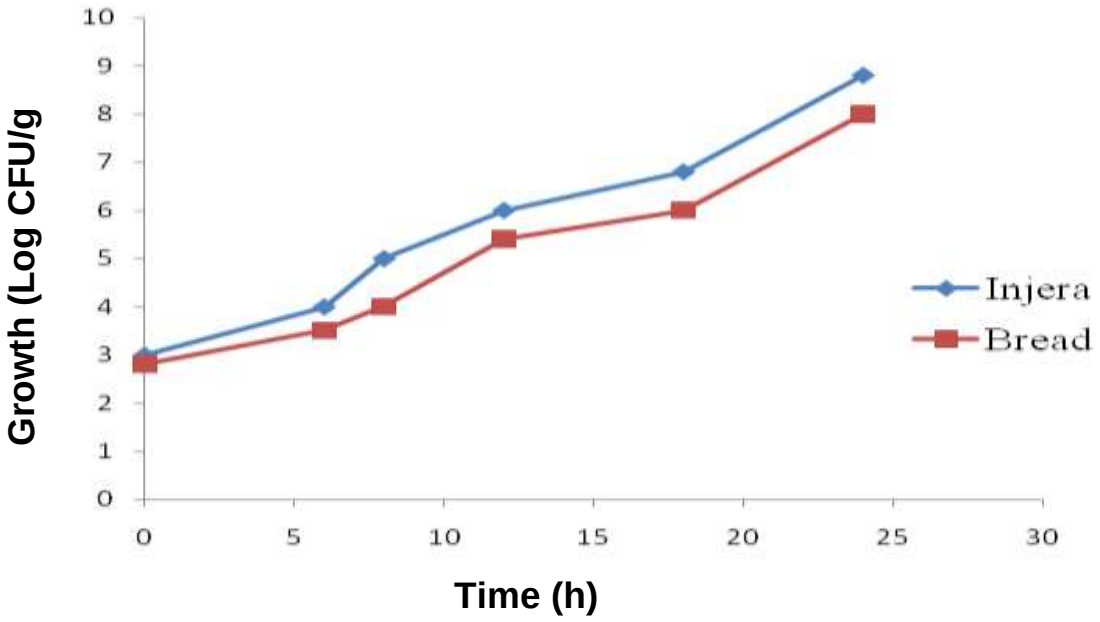

Figure 2. The growth potential of *Salmonella* spp. isolated from mobile phones, Jimma town, Southwest Ethiopia.

Table 1. Antimicrobial susceptibility patterns of *Salmonella* spp. isolated from mobile phones, Jimma town, Southwest Ethiopia.

Antimicrobial discs with defined concentration (µg)	Resistance (%)	Intermediate (%)	Susceptible (%)
Ciprofloxacin (5)	-	-	22 (100)
Ampicillin (10)	21 (95.5)	-	1 (4.55)
Chloramphenicol (30)	-	2 (9.09)	20 (90.91)
Nalidixic acid (30)	18 (81.82)	2 (9.09)	2 (9.09)
Kanamycin (30)	-	3 (13.64)	19 (86.36)
Norfloxacin (10)	-	-	22 (100)
Gentamycin (10)	-	1 (4.55)	21 (95.45)
Tetracycline (30)	6 (27.27%)	-	16 (72.73)
Streptomycin (10)	15 (68.18)	3 (13.64)	4 (18.18)

Table 2. Antimicrobial susceptibility patterns of *S. aureus* isolated from mobile phones, Jimma town, Southwest Ethiopia.

Antimicrobial discs with defined concentration	Resistance (%)	Intermediate (%)	Susceptible (%)
Gentamycin(10)	-	2 (2.6)	76 (97.4)
Erythromycin(15)	31 (39.7)	34 (43.6)	13 (16.67)
Chloramphenicol(30)	1 (1.3)	2 (2.6)	75 (96.1)
Ciprofloxacin(5)	3 (3.8)	2 (2.6)	73 (93.6)
Amikacin(30)	1 (1.3)	2 (2.6)	75 (96.1)
Kanamycin(30)	2 (2.6)	6 (7.7)	70 (89.7)
Streptomycin(10)	-	6 (7.7)	72 (92.3)
Penicillin G (10)	78 (100)	-	-
Tetracycline(30)	21 (26.9)	32 (41.035)	25 (32.05)
Clindamycin(2)	57 (73.1)	17 (21.8)	4 (5.1)

DISCUSSION

The current challenge studies conducted using *Salmonella* spp. isolated from mobile phones of food handlers and health care workers revealed that the isolates grew to their infective doses (≥5 Log CFU/g) in bread and injera samples within 12 and 18 h, respectively. The maximum counts recorded were ≥8 Log CFU/g in both food items (Bread and Injera) within 24 h.

Likewise, report from Addis Ababa Ethiopia made by Muleta and Ashenafi (2001) indicated that the mean counts of *Salmonella* isolates reached >8 Log CFU/g within 24 h. On the other hand, Erku and Ashenafi (1998) evaluated the growth potential of *Salmonella* spp. in weaning foods in Addis Ababa, Ethiopia where *Salmonella* had grown to approximately 4 Log CFU/ml within 8 h and reached counts as high as Log 8 CFU/ml within 12 h.

S. aureus isolates tested in the present study had reached mean counts≥6 Log CFU/g in both food items (Bread and Injera) within 12 and18 h, respectively. The infective dose for *S. aureus* is 6 Log CFU/g. This is in agreement with the study reported by Muleta and Ashenafi (2001) from Addis Ababa Ethiopia. *S. aureus* toxin is produced when the count exceeds 6 Log CFU/g. The maximum growth of *S. aureus* (9 Log CFU/g) was observed in the current study within 24 h. In general, the trends of growth of the test strains were similar with increasing pattern almost throughout the observation period. *Salmonella* spp. isolated from mobile phones indicates marked resistance to commonly used antibiotics, such as ampicillin (95.45%), nalidixic acid (81.82%), and streptomycin (68.18%). Similarly, in a study conducted in Bangladesh, Ahmed et al. (2011) reported higher frequency of *Salmonella* spp. resistant to ampicillin and nalidixic acid. In the current study, however, *Salmonella* spp. isolates were found sensitive to ciprofloxacin (100%), norfloxacin (100%), gentamycin (95.45%), chloramphenicol (90.91%), and kanamycin (86.36%). Likewise, the highest frequency (100%) of sensitivity to chloramphenicol was reported in earlier studies from India (Cailhol et al., 2005; Nesa et al., 2011). *Salmonella* spp. isolate has shown resistance against five antibiotics in this study which were considered as multidrug resistant strains (Sivakumar et al., 2012). The major reasons for the presence of multidrug resistance among *Salmonella* spp. is due to mutability of bacteria and inappropriate use of antibiotics (Ochman et al., 1996). Many people purchases antibiotics in the open market without any medical prescription and use them for the wrong diseases and infections (Tagoe and Attah, 2010).

S. aureus resistance to antibiotics such as penicillin g (100%), clindamycin (94.4%), tetracycline (76.9%), and erythromycin (74%) in this study is in agreement with the study by Tambekar et al. (2008) from India where the highest frequency of resistance was recorded for antibiotics penicillin, erythromycin, and tetracycline. This indicates that there might be fast growing public health threat within the community. Therefore, it requires strong controlling system of the personal hygiene and educating food handlers and health care workers regarding microbial contamination of mobile phones. Mohamad et al. (2010) from Cairo reported that, *S. aureus* isolated from mobile phones of health care workers demonstrated the highest frequency of resistance to several antimicrobials. This may be due to indiscriminate use of multiple antibiotics, prolonged hospital stay, intravenous drug abuse, self-medication, and inappropriate use of antibiotics (Tagoe and Attah, 2010). *S. aureus* isolate had shown multidrug resistance against six antibiotics. This could be the major challenge for treating staphylococcal infections.

In conclusion, *Salmonella* spp. and *S. aureus* isolated from mobile phones of food handlers and health care workers in Jimma town, Southwest Ethiopia were found to be able to grow to their infective doses within 12 to18 h in both food items (Bread and Injera). In addition, both *Salmonella* spp. and *S. aureus* isolates had shown the highest frequency of resistance for most of the antimicrobials tested. This indicates that mobile phones could play a significant role in spreading drug resistant infectious agents within the community. Therefore, the outmost care should be taken in using mobile phones.

Conflict of Interests

The authors have not declared any conflict of interests.

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Anti-bacterial, anti-oxidant and cytotoxicity of aqueous and organic extracts of *Ricinus communis*

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The present study aimed to examine anti-microbial, anti-oxidant and cytotoxicity in leaf extract of *Ricinus communis* extract (in different solvent). The leaf powder of *R. communis* was extracted using different solvents. The anti-bacterial activity of the extracts was determined by agar well and disc diffusion method. The extracts were also subjected to phytochemical analysis. The anti-oxidant activity of the extracts was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), alkaline DMSO, deoxyribose and nitric oxide scavenging assay. The cytotoxicity of the extracts was estimated using MTT cell proliferation assay. The photochemical qualitative analysis of methanollic plant extracts revealed the presence of alkaloid, flavonoid, tannins, glycoside, reducing sugar, anthraquinones and saponins. The methanollic extract showed zone of inhibition of 15 mm each against *Bacillus subtilis, Staphylococcus epidermis* and *Saccharomyces cereviceae* by using well diffusion method, whereas *S. cereviceae* gave 12 mm zone of inhibition by disc diffusion at a concentration of 40 mg/mL. The anti-oxidant activity by different methods gave IC_{50} value of 102.1 ± 4.16, 30.27 ± 3.85 and 382.6 ± 3.30 µg/mL in aqueous, benzene and ethyl acetate extract respectively by using DPPH method. The acetone extract gave IC_{50} value of 357.1 ± 4.96 µg/mL by nitric oxide method. The aqueous and acetone extract gave IC_{50} value of 860.1 ± 7.73 and 626.7 ± 2.25 µg/mL, respectively by deoxyribose method. The chloroform and ethyl acetate extract showed cytotoxicity in A549 cell line having IC_{50} value of 687 ± 3.92 and 957 ± 4.46 µg/mL respectively by MTT cell proliferation assay whereas, aqueous extract in Jurkat cell line gave IC_{50} value of 918 ± 2.05 µg/mL. This study demonstrates that the *R. communis* extracts are potential source for anti-microbial, anti-oxidant and anti-cancer agent. Further study is needed to identify the specific bioactive compounds, their mode of action and their non-toxic nature in *in vivo* condition.

Key words: *Ricinus communis*, (3-(4, 5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide) (MTT) assay and 1,1-diphenyl-2-picrylhydrazyl (DPPH).

INTRODUCTION

The herbal medicines and products have been used in the pharmaceuticals company for the production of medicines since time unknown. *Ricinus communis* (Euphorbiaceae) is commonly known as Arand in India.

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The name Arand indicates the property of the plant to drive out diseases. The castor oil is a reputed remedy for all kinds of rheumatic affections. It has been reported to cure dyspnoea, hydrocele, flatulence, dysentery, ascites, piles, cough, lumbago, headache, leprosy, arthritis, calculus, disuria, elevates phantom tumor, spleen disorders, impurity of blood, dyspepsia and worm troubles (Jena and Gupta, 2012). The studies carried out by various workers have shown anti-microbial activity against *Salmonella typhimurium*, *Proteus vulgaris*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus niger*, *Pseudomonas aeruginosae*, *Escherichia coli* (Jombo and Enenebeaku, 2008; Kota and Manthri 2011; Verma et al., 2011; Dastagir et al., 2012; Khursheed et al., 2012; Kensa and Yasmin, 2011) and *Enterococcus faecalis* (Lekganyane et al., 2012) and anti-oxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and reducing power assay (Singh et al., 2010; Kadri et al., 2011). The n-butanol extract of *R. communis* showed 0.625 and 2.50 µg/mL anti-bacterial activities against Gram positive bacteria *Staphylococcus aureus* and Gram negative bacteria *Shigella flexneri* respectively (Iqbal et al., 2012). A wide spectrum of *R. communis* as an anti-microbial agent has been reported. It has found to inhibit secondary infection in oral cancer patients (Panghal et al., 2011). In one of the study using *R. communis,* leaf extract showed zone of inhibition of 15.90 ± 2.13 mm against *Staphylococcus aureus*. The MIC values against the strain ranged from 1.95 to 250 mg/mL for different leaves extracts (Bereket et al., 2014). The hot and cold methanol and ethanol extracts showed inhibition on both *S. aureus* and *E. coli*.

The hot and cold ethanol extracts revealed inhibition of *S. aureus* with MIC and MBC values of 5 and 10 mg/mL, respectively. *E. coli* was inhibited by hot extracts of both ethanol and methanol having the MIC and MBC values of 40 and 80 mg/mL, respectively (Jeyaseelan and Jashothan, 2012). The *R. communis,* petroleum ether and acetone extracts showed anti-microbial activities against dermatophytic and pathogenic bacterial strains, *Streptococcus progenies, Staphylococcus aureus* as well as *Klebsiella pneumoniae* and *Escherichia coli* (Islam et al., 2010). The hexane and ethanolic leaf extract of *R. communis* also showed anti-bacterial activity against *B. subtilis* and *E. coli* (Bais, 2014). The phytochemicals alkaloids and cardiac glycosides were found in high concentration in the leaves and stem extracts of *R. communis.* It was found to give 11.2 and 63.60% yields respectively and was also responsible for its antioxidant and anti-hemolytic activities. Furthermore, extracts of these two phytochemicals also showed a decrease in the growth and proliferation of pathogenic *Klebsiella pneumonia* and *Staphylococcus aureus* (Ibraheem and Maimako, 2014). The essential oil of *R. communis* showed cytotoxicity in HeLa cell lines and anti-microbial activity against *B. subtilis*, *S. aureus* and *Enterobacter cloacae* (Zarai et al., 2012).

MATERIALS AND METHODS

Collection and identification of plant

The leaves of plant were collected from in and around the campus of Integral University, Lucknow, India. The plants were authenticated and sample vouchers were stored in NBRI, Lucknow. The identified plant parts were washed and air dried at room temperature and was powdered with the help of mortar and pestle. The plant extracts prepared using Soxhlet apparatus in different solvents.

Plant extracts preparation using different solvents

A 20 g of finely ground dry plant parts were weighed and kept in a muslin cloth. The muslin cloth containing dry extract were placed in extraction chamber of the soxhlet apparatus. The extraction solvent in boiling flask was heated and its vapour condenses in the condenser. The condensed solvent strips into the thimble containing the crude dry plant and extracts it by contact. When the level of liquid in extraction chamber rises to the top of siphon tube, the liquid contents of thimble siphon in flask A. This process is continous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated (Handa et al., 2008). The aqueous and organic solvents were used for crude extract preparation. The organic solvents used in the present study were cyclohexane benzene, chloroform, acetone, ethyl acetate, ethanol and methanol. All the extracts were air dried in petri plates and the extracts were weighed and kept in eppendroff at -20° C until used.

Determination of extraction yield in plant extract (% yield)

The yield (% w/w) from all dried extracts was calculated by the formula:

$$\text{Yield (\%)} = \frac{W_2 - W_1}{W_0 \times 100}$$

Where, W_2 is the weight of the extract and the container, W_1 weight of the container alone and W_0 the weight of the plant powder (Anokwuru et al., 2011).

Phytochemical analysis (qualitative) of plant extract

The qualitative phytochemical analysis was estimated on the basis of color formation by standard methods (Tiwari et al., 2011; Mir et al., 2013; Trease and Evans, 1983; Kokate et al., 1997; Hegde and Joshi, 2010). They are as follows:

Alkaloids

The plant extract was prepared by taking 500 mg of dry plant material in 500 mL of methanol on a water bath at 37°C for 20 min, the extract was filtered and allowed to cool and few drops of Wagner's reagent added (2 g iodine and 6 g of potassium iodide in 100 mL distilled water). A reddish brown colored precipitate indicated the presence of alkaloids.

Anthraquinones (Borntrager's test)

The 0.5 g of dry plant material was boiled with 10% hydrochloric acid (HCl) in a water bath, filtered and allowed to cool and equal volume of chloroform ($CHCl_3$) and few drops of 10% ammonia was

added and heated. Formation of rose-pink color indicated the presence of anthraquinones.

Flavonoids

The crude methanolic extract was heated with 10 mL of ethyl acetate for 3 min, filtered and 1 mL of ammonia solution was added to 4 mL filtrate, formation of yellow color indicated the presence of flavonoids.

Phlobatannins

An aqueous extract of plant sample was boiled with 1% aqueous hydrochloric acid (HCl) and the deposition of red precipitate showed the presence of phlobatannins.

Glycosides (Fehling's test)

To the 2 mL of methanolic extract, 10 mL of 50% hydrochloric acid (HCl) was added and heated in water bath for 30 min and then 5 mL of Fehling's solution was added. After 5 min, formation of brick red precipitate indicated the presence of glycosides.

Saponins (Frothing test)

The 0.2 g of the methanolic extract was shaken with 5 mL of distilled water and then heated to boil. Frothing (appearance of creamy mist of small bubbles) showed the presence of saponins.

Steroids (Salkowski test)

The methanolic extract was dissolved in methanol and to it 5 drops of concentrated sulphuric acid (H_2SO_4) was added. The formation of red color indicated the presence of steroids.

Tannins (Ferric chloride test)

The 0.5 g of methanolic extract was dissolved in 10 mL of distilled water, filtered and ferric chloride reagent was added, a blue-black precipitate was taken as evidence for the presence of tannin.

Terpenoids (Salkowski test)

The 0.2 g of dried plant material was mixed with 2 mL of chloroform and 3 mL of concentrated hydrochloric acid (HCl) was added carefully to form a layer. A reddish brown coloration of the interface formed indicated the presence of terpenoids.

Reducing sugar

The 0.5 g of methanolic extract dissolved in 1 mL of distilled water and 2 to 8 drops of Fehling solution was added and boiled for few minutes. The presence of brick red precipitate indicated presence of reducing sugar.

Anti-microbial activity

The anti-microbial activity was screened in extracts by disc diffusion and well diffusion method. The bacterial strains used were *S. aureus* 2079, *E. coli* 2065, *Proteaus vulgaris* 2027, *B. cereus* 2156, *B. subtilis* 296, *S. epidermis* 2493 and *S. cereviceae* 3090. All the strains were obtained from the National Chemical Laboratory (NCL), Pune, India. Dried filter paper discs (4 mm) impregnated in known amount of test samples and for well diffusion method, the extracts were inoculated in well prepared using well cutter (0.6 cm). The dried plant extracts were prepared in dimethyl sulfoxide at a concentration of 40, 30, 20, 10 and 5 mg/mL. The plates were incubated at 37° C for 24 h. Anti-microbial activity was determined by measuring the diameter of zone of inhibition. For each bacterial strain, controls were maintained in which dimethyl sulfoxide was used as a negative control and the discs of Tetracycline (30 mcg/disc), Penicillin G (10 units/disc), Streptomycin (10 mcg/disc) and Amoxicillin (30 mcg/disc) were used as a positive control. The experiment was done three times and the mean values were presented (Kamaraj et al., 2012).

Anti-oxidant assay

The different anti-oxidant assays were used for the study of DPPH method, superoxide radical with the alkaline DMSO (dimethyl sulfoxide) method, nitric oxide radial inhibition assay and hydroxyl radical in the deoxyribose method. L-ascorbic acid, butylated hydroxy toluene and quercetin were used as standard, while methanol or dimethyl sulfoxide was used in place of plant extract as control. The crude plant extracts were prepared at different concentrations varying from 1000 µg/mL to 0.46 µg/mL.

DPPH (1, 1 – Diphenyl – 2- Picryl Hydrazyl) radical scavenging activity method

DPPH radical scavenging activities of all the fractions were determined by the method of Blois (1958) with some modification. The crude plant extracts of 10 µl was mixed with 200 µl of 100 mM DPPH (dissolved in methanol). The reaction mixtures were incubated for 30 min at 37°C under dark condition. The absorbance was measured at 490 nm spectrophotometrically (Ara and Nur, 2009).

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of extract)}}{\text{Absorbance of control}} \times 100$$

Scavenging of superoxide radical with the alkaline DMSO (Dimethyl sulfoxide) method

Alkaline DMSO radical scavenging assay were determined by the method of Kunchandy and Rao (1990) with slight modification (Sanja et al., 2009; Vaijanathappa et al., 2008). The reaction was prepared by mixing 0.1 mL of nitro blue tetrazolium (1 mg/mL in DMSO) and 1 mL of alkaline DMSO (1 mL of DMSO containing sodium hydroxide 5 mM in 0.1 mL of water). To the reaction mixture 0.3 mL of the crude extract prepared in DMSO was added. The absorbance was measured at 560 nm spectrophotometrically.

$$\text{Percentage super oxide scavenging activity} = \frac{\text{Test absorbance} - \text{Control absorbance}}{\text{Test absorbance}} \times 100$$

Anti-oxidant assay by nitric oxide radial inhibition assay

The plant extracts (1 mL) was mixed with 1 mL phosphate buffer saline and 4 mL (10 mM) sodium nitroprusside and was kept for incubation at room temperature at 25°C for 150 min. After incubation,

0.5 mL of reaction mixture and 1 mL sulphanilic acid reagent (0.33% sulphanilic acid in 20% glacial acetic acid) were added and incubated for 5 min at room temperature (for diazotization reaction). Then 1 mL N-(1-naphthyl) ethylene-di-amine di-hydrochloride was added and kept in diffused light for 30 min and absorbance was measured at 540 nm (Badami et al., 2005).

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$$

Scavenging of hydroxyl radical in the deoxyribose method

The scavenging of hydroxyl free radical was measured by the method of Halliwell et al. (1987) with minor changes. The reaction mixture prepared containing deoxyribose (3 mM) 0.2 mL; ferric chloride (0.1 mM) 0.2 mL; ethylene diamine tetra acetic acid disodium salt (EDTA) (0.1 mM) 0.2 mL; ascorbic acid (0.1 mM) 0.2 mL and hydrogen peroxide (2 mM) 0.2 mL in phosphate buffer (pH, 7.4, 20 mM). To the reaction mixture, 0.2 mL of various concentrations of the extract or standard in DMSO was added to form a final volume of 1.2 mL. The solution was then incubated for 30 min at 37°C. After incubation, ice-cold tri-chloro acetic acid (0.2 mL, 15% w/v), and thio-barbituric acid (0.2 mL, 1% w/v) in 0.25 N hydrochloric acid were added. The reaction mixture was then kept in a boiling water bath for 30 min, cooled, and the absorbance was measured at 532 nm (Hinneburg et al., 2006).

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$$

Cytotoxicity by MTT cell proliferation assay

Cell proliferation were measured by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide) assay that colorimetrically measures a purple formazan compound produced by viable cells (Mosmann, 1983). The cell lines used for the present study were Jurkat (human lymphoblastic leukeamia), Hek 293 (human kidney), A549 (human alveolar adenocarcinoma) and MRC-5 (human lung). The cells were plated 0.5 x 10^4 cells for A549, Hek 293 and 1 x 10^4 cells for MRC-5 and Jurkat in 96 well plates. After 24 h of plating, cells were treated with crude plant extracts of different solvents at the concentrations of 1000, 500, 250, 125 and 62.5 µg/mL. The treated cells were incubated for 24 h at 37°C. After 24 h of treatment, MTT was added and again the cells were kept for 4 h at 37°C. The formation of purple formazan compound produced by viable cells was dissolved in dimethyl sulphoxide and the plates were read at 570 nm wavelength using ELISA reader. All the assays were performed in triplicate. The percentage inhibition was calculated in cancerous and normal cell lines and IC_{50} values were determined.

RESULTS

The present study shows that medicinal plants possess anti-microbial, anti-oxidant and cytotoxic properties that support *R. communis* value in herbal medicine for the treatment of different diseases. The presence of alkaloid, flavonoid, tannins, glycoside, reducing sugar, anthraquinones and saponins which were estimated qualitatively were found and may be responsible for its anti-microbial, anti-oxidant and cytotoxicity of *R. communis* extracts. The initial weight of the dried plant was taken as 20 g in 200 mL of solvent. The percentage yield in aqueous extract is 10.7%, cyclohexane extract 0.9%, benzene extract 1.85%, chloroform extract 3.5%, acetone extract 6.15%, ethyl acetate extract 0.9%, ethanol extract 0.4% and methanol extract 0.9%. A wide range of the yields among extracts was observed depending on the extraction solvent.

Anti-microbial activity of *R. communis* extract

The *R. communis* aqueous and organic extracts showed significant anti-microbial activity against *B. subtilis, E. coli*, *S. epidermis*, *S. cereviceae*, *P. vulgaris*, *B. cereus* and *S. aureus*. The anti-bacterial activity by well diffusion method was found to be in the order of methanol > aqueous > benzene > ethyl acetate > acetone extract. The cyclohexane, chloroform and ethanolic extracts did not show activity against any of the strains used. The results of anti-microbial activity of *R. communis* showing zone of inhibition by well diffusion method are given in Table 1. The disc diffusion method showed anti-bacterial activity in the order of methanol > ethyl acetate > aqueous > benzene > acetone extract whereas, cyclohexane, chloroform and ethanol extract did not show anti-microbial activity against any of the strains used. The results of anti-microbial activity of *R. communis* showing zone of inhibition by well diffusion method are given in Table 2 and Figure 1. The plant extracts were compared with the standard antibiotics as a positive control and dimethyl sulphoxide (DMSO) as negative control against different bacterial strains. The plant extracts when compared with the antibiotics for their anti-bacterial activity showed significant activity and zone of inhibition in them were found to be equivalent to the standard anti-biotics. The zone of inhibition of standard antibiotics and negative control dimethyl sulfoxide are given in Table 3.

Comparative IC_{50} values of aqueous and organic extracts by different anti-oxidant assay

The IC_{50} values of the aqueous and organic extracts were calculated by different anti-oxidant assay that is DPPH, alkaline DMSO, nitric oxide scavenging assay, and hydroxyl radical assay in the deoxyribose method. The aqueous and organic extracts of *R. communis* showed 50% inhibition against the above mentioned anti-oxidant assays as shown in Table 4, Figures 2, 3, 4 and 5. The results given show that benzene extract of *R. communis* with IC_{50} value of 30.27 ± 3.85 µg/mL possesses strong anti-oxidant activity as compared to other extracts used for the present study.

Cytotoxicity in different cell lines by crude plant extracts using MTT assay

R. communis aqueous leaf extract showed maximum

Table 1. Zone of inhibition (mm) of *Ricinus communis* extract in different solvents by agar well diffusion method.

Plant extract	Conc. of extract (mg/mL)	Zone of Inhibition (mm)						
		B. subtilis	***E. coli***	***S. epidermis***	***S. cereviceae***	***P. vulgaris***	***B. cereus***	***S. aureus***
Aqueous	40	13 ±2.33	NZ	14±0.5	NZ	NZ	9 ±1	NZ
	30	12 ±4.51	NZ	13 ±0.55	NZ	NZ	6 ±4.44	NZ
	20	11 ±3.78	NZ	12 ±0.05	NZ	NZ	4 ±3.21	NZ
	10	10 ±0	NZ	11 ±0.05	NZ	NZ	2 ±0	NZ
	5	2 ±0	NZ	NZ	NZ	NZ	NZ	NZ
Cyclohexane	40	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	20	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Benzene	40	NZ	NZ	13 ±0	NZ	NZ	NZ	9 ±0.11
	30	NZ	NZ	12 ±2.56	NZ	NZ	NZ	6 ±0.1
	20	NZ	NZ	11 ±1.09	NZ	NZ	NZ	4 ±0
	10	NZ	NZ	10 ±0.1	NZ	NZ	NZ	2 ±5.6
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Chloroform	40	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	20	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Acetone	40	NZ	NZ	NZ	NZ	NZ	NZ	9 ±5.5
	30	NZ	NZ	NZ	NZ	NZ	NZ	8 ±0
	20	NZ	NZ	NZ	NZ	NZ	NZ	6 ±1
	10	NZ	NZ	NZ	NZ	NZ	NZ	4 ±0
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Ethyl acetate	40	16 ±0.5	NZ	NZ	NZ	NZ	NZ	12 ±1
	30	15 ±0.1	NZ	NZ	NZ	NZ	NZ	10 ±1.2
	20	14 ±0.5	NZ	NZ	NZ	NZ	NZ	9 ±0
	10	12 ±0.5	NZ	NZ	NZ	NZ	NZ	8 ±4.5
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Ethanol	40	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	20	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Methanol	40	15 ±0	12 ±1	15 ±0.1	15 ±4.4	NZ	10 ±0.5	NZ
	30	14 ±2.33	10 ±0.9	14 ±4.55	14 ±2.30	NZ	8 ±0.05	NZ
	20	13 ±0.1	8 ±0.1	13 ±6.0	13 ±0	NZ	7 ±0.05	NZ
	10	12 ±0.5	7 ±0.2	12 ±2.33	12 ±3.1	NZ	5 ±0	NZ
	5	9 ±0.5	NZ	4 ±0	2 ±9.08	NZ	NZ	NZ

The bacterial strains used were *B. subtilis*, *E. coli*, *S. epidermis*, *S. cereviceae*, *P. vulgaris*, *B. cereus* and *S. aureus* at the concentration of 40, 30, 20, 10 and 5 mg/mL, respectively (NZ- No Zone). The values are mean ± standard deviation (n=3).

Table 2. Zone of inhibition (mm) of *Ricinus communis* extract in different solvents by disc diffusion method.

Plant extract	Conc. of extract (mg/ml)	Zone of Inhibition (mm)						
		B. subtilis	***E. coli***	***S. epidermis***	***S. cereviceae***	***P. vulgaris***	***B. cereus***	***S. aureus***
Aqueous	40	10 ± 0	NZ	12 ± 0.08	NZ	NZ	NZ	NZ
	30	9 ± 0.2	NZ	10 ±0.03	NZ	NZ	NZ	NZ
	20	8 ± 0.2	NZ	8 ± 0	NZ	NZ	NZ	NZ
	10	NZ	NZ	6 ± 0.22	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Cyclohexane	40	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	20	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Benzene	40	NZ	NZ	10 ± 0	NZ	NZ	NZ	NZ
	30	NZ	NZ	9 ± 3.2	NZ	NZ	NZ	NZ
	20	NZ	NZ	7 ± 3.35	NZ	NZ	NZ	NZ
	10	NZ	NZ	4 ± 0.5	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Chloroform	40	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	20	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Acetone	40	NZ	NZ	NZ	NZ	NZ	NZ	9 ± 0.45
	30	NZ	NZ	NZ	NZ	NZ	NZ	8 ± 0.21
	20	NZ	NZ	NZ	NZ	NZ	NZ	6 ± 5.0
	10	NZ	NZ	NZ	NZ	NZ	NZ	4 ± 1.30
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Ethyl acetate	40	12 ± 3.2	NZ	NZ	NZ	NZ	NZ	12 ± 0
	30	10 ± 3.4	NZ	NZ	NZ	NZ	NZ	10 ± 3.2
	20	9 ± 7.5	NZ	NZ	NZ	NZ	NZ	9 ± 1.2
	10	NZ	NZ	NZ	NZ	NZ	NZ	8 ± 0
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Ethanol	40	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	20	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Methanol	40	10 ± 0.05	7 ± 0.5	NZ	12 ± 3.22	NZ	8 ± 0	NZ
	30	9 ± 0.5	4 ± 0.5	NZ	10 ± 0	NZ	8 ± 1	NZ
	20	6 ± 0.5	2 ± 0.1	NZ	8 ± 0.5	NZ	7 ± 0.5	NZ
	10	4 ± 0.1	NZ	NZ	5 ± 0.05	NZ	5 ± 0.2	NZ
	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ

The bacterial strains used were *B. subtilis*, *E. coli*, *S. epidermis*, *S. cereviceae*, *P. vulgaris*, *B. cereus* and *S. aureus* at the concentration of 40, 30, 20, 10 and 5 mg/mL, respectively (NZ- No Zone). The values are mean ± standard deviation (n=3).

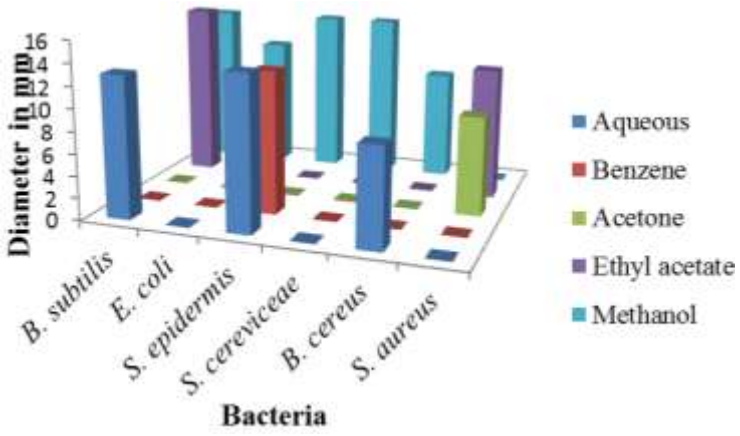

Figure 1. Comparative anti-microbial activity in *R. communis* extracts against selected bacteria.

Table 3. The data represents zone of inhibition (mm) of the standards. The antibiotics used as a positive control are Amoxicillin, Penicillin G, Tetracycline and Streptomycin. Dimethyl sulphoxide was used as a negative control. The values are mean ± standard deviation (n=3).

Standards	*B. subtilis*	*E. coli*	*S. epidermis*	*S. cereviceae*	*P. vulgaris*	*B. cereus*	*S. aureus*
Amoxicillin	NZ	NZ	12 ±3.21	NZ	NZ	NZ	NZ
Penicillin G	NZ	NZ	NZ	NZ	20 ±0.91	NZ	NZ
Tetracycline	27 ± 0.05	24 ± 0.1	28 ±0	23 ±0.2	25 ±0.43	16 ±0.5	25 ±0.1
Streptomycin	20 ±0.5	16 ±0	NZ	20 ±0.05	20 ±0.1	21 ±0.63	15 ±0.11
Dimethyl sulfoxide	NZ	NZ	NZ	NZ	NZ	NZ	NZ

Table 4. Comparative chart of IC_{50} values of aqueous and organic extracts of *Ricinus communis* and standard L-ascorbic acid, BHT and quercetin. The plant extracts given were *R. communis* Unit for IC_{50} for all the activities are µg/mL. Data are expressed as mean ± SD (n=3). *p< 0.0001 vs 0 µg/mL.

Plant name	Extract in different solvents	IC_{50} values ± SD (µg/mL) of different anti-oxidant assay			
		DPPH	Alkaline DMSO	Nitric oxide	Deoxyribose
R. communis	Aqueous	102.1±4.16	-	-	860.1±7.73*
	Benzene	30.27±3.85	-	-	-
	Acetone	-	-	357.1±4.96*	626.7±2.25*
	Ethyl acetate	382.6±3.30*	-	-	-
L-ascorbic acid		61.4±1.55	537.7±14.33	54.97±4.73	865.2±1.50*
BHT		50.8±3.85	801.5±0	461.3±2.54*	958.8±0
Quercetin		27.9±1.55	316.5±1.21*	47.57±10.68	419.9±1.2*

cytotoxicity to Jurkat cells at a concentration of 1000 µg/mL, as compared to other extracts used whereas, the chloroform and ethyl acetate extracts showed maximum cytotoxicity on A549 cell line (Figures 6 and 7). The IC_{50} values of different extracts are listed in Table 5. The cytotoxicity on Jurkat and A549 cell lines indicates the anti-cancer activity of the crude plant extracts. The most significant activity against A549 cell line was showed by chloroform extract of *R. communis.*

DISCUSSION

In the present study, *R. communis* extracts (using different solvents) were tested to determine their inhibitory effect against standard bacteria, *S. aureus*, *E. coli, P. vulgaris, B. cereus, B. subtilis, S. epidermis* and *S. cereviceae*. The results demonstrated that these extracts had ability to control the bacteria *in vitro*. Different organic solvents beside aqueous solution were

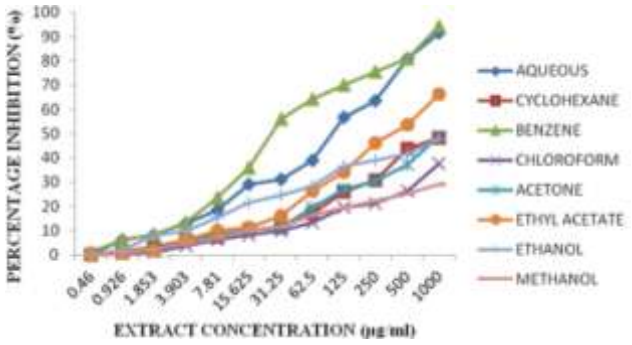

Figure 2. The scavenging effect of aqueous and organic extracts of *Ricinus communis* by DPPH method. The different concentrations of extracts used were 1000 to 0.46 µg/mL. The data represent the percentage DPPH inhibition Values are expressed as mean ± SD (n=3).

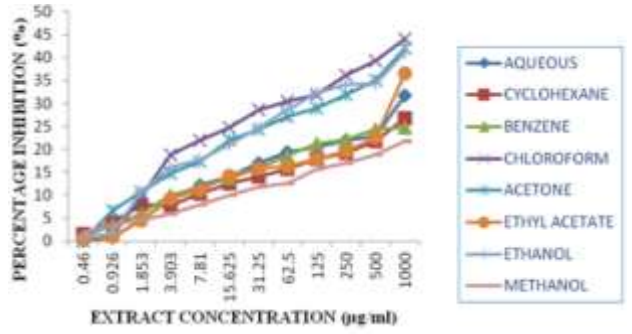

Figure 3. The scavenging effect of aqueous and organic extracts of *Ricinus communis* by Alkaline DMSO method. The different concentrations of extracts used were 1000 to 0.46 µg/mL. The data represent the percentage alkaline DMSO inhibition. Values are expressed as mean ± SD (n=3).

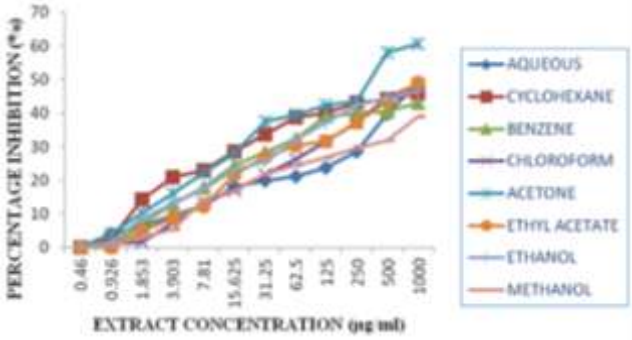

Figure 4. The nitric oxide radical scavenging activity of aqueous and organic extracts of *Ricinus communis*. The different concentrations of extracts used were 1000 to 0.46 µg/mL. The data represent the percentage nitric oxide inhibition. Values are expressed as mean ± SD (n=3).

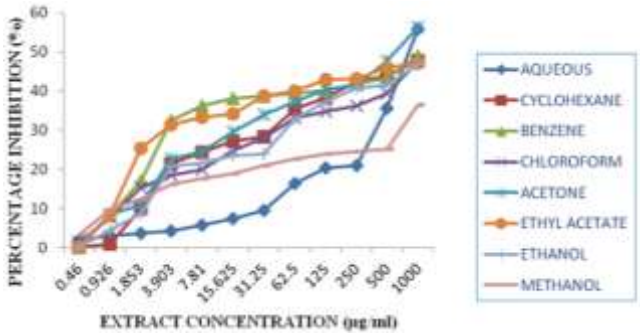

Figure 5. The hydroxyl radical scavenging activity of aqueous and organic extracts of *Ricinus communis* by deoxyribose method. The different concentrations of extracts used were 1000 to 0.46 µg/mL. The data represent the percentage inhibition values. Values are expressed as mean ± SD (n=3).

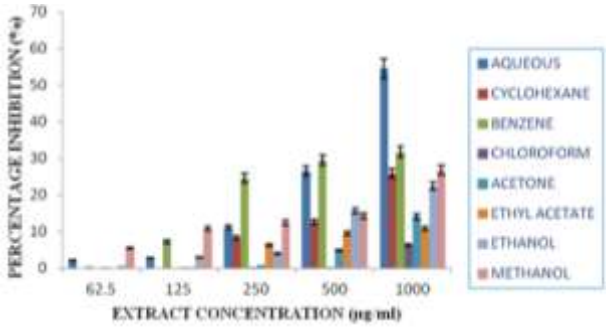

Figure 6. The cytotoxic effect of *Ricinus communis* extracts on Jurkat cell line using MTT assay. The different concentrations of extracts used were 1000 to 62.5 µg/mL. The data represent the percentage (%) inhibition. Values are expressed as mean ± SD (n=3).

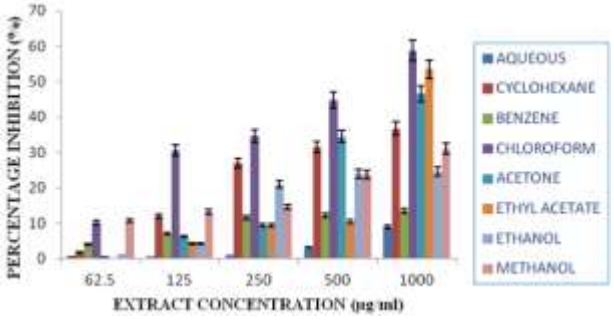

Figure 7. The cytotoxic effect of *Ricinus communis* extracts on A549 cell line using MTT assay. The different concentrations of extracts used were 1000 to 62.5 µg/mL. The data represent the percentage (%) inhibition. Values are expressed as mean ± SD (n=3).

used for extraction, but methanol extract showed maximum anti-microbial activity when compared to the other extracts. The methanol and ethanol are polar solvents but with different polarity, methanol has higher polarity than ethanol. The polarity of the solvents deter-

Table 5. Comparative chart of IC_{50} values of different cancerous and normal cell lines.

Plant name	Extract in different solvents	Jurkat	Hek-293	A-549	MRC-5
Ricinus communis	Aqueous	918±2.05* µg/mL	-	-	-
	Chloroform	-	-	687±3.92* µg/mL	-
	Ethyl acetate	-	-	957±4.46* µg/mL	-

The concentrations of plant extract which reduced cell viability of cell lines to 50% were given in µg/mL. Data represents the mean ± SD (n=3). p <0.0001* versus 0 µg/mL.

mines the solubility of chemicals from plant powder (El-Mahmood and Doughari, 2008). The high polarity of methanol extract was found to be more effective against Gram positive bacteria *B. subtilis* and *S. aureus*, as well as Gram negative bacteria *P. aeruginosae* and *K. pneumoniae* as compared to ethanol and aqueous extracts (Naz and Bano, 2012). The Gram positive bacteria show more sensitivity to biomolecules present in plant extracts than the Gram negative bacteria. This difference is due to the cell wall composition of the two bacteria (Panda et al., 2009). The anti-bacterial activity of *R. communis,* reported earlier, against two strains that is *Enterobacter* sp. and *Bacillus subtilis* also correlated with the present study (Rao et. al., 2013). The n-hexane, chloroform, ethyl acetate and n-butanol extracts of *R. communis* also showed anti-bacterial activity against Gram positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis* as well as Gram negative bacteria, *E. coli* and *Shigella flexneri* (Iqbal et al., 2012).

The photochemical qualitative analysis of methanolic extracts showed the presence of alkaloid, flavonoid, tannins, glycoside, reducing sugar, anthraquinones and saponins. However, in the present study, the methanol extracts showed microbial growth inhibition in both agar well diffusion method and disc diffusion method. The presence of phytochemicals in the plants is responsible to protect them from infection of pathogenic micro-organisms (Cowan, 1999). Recent studies on biological activity of phytochemicals have demonstrated the value of phytochemicals in drug discovery. Flavonoids are hydroxylated phenolic substances and they are known to be synthesized by plants in response to microbial infection. Their activity is probably due to their ability to form complex with extracellular and soluble proteins and also with bacterial cell wall. The more lipophilic flavonoids may also disrupt microbial membranes (Cowan, 1999). Saponins interfere with or alter the permeability of the cell wall while the tannins act by coagulating the cell wall proteins (Jeyaseelan and Jashothan, 2012). The presence of saponins in *R. communis* is responsible for its anti-oxidant, anti-cancer and folklore remedies (Vandita et al., 2013). Polyphenols are anti-oxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers and some of the polyphenols also show metal chelation properties (Proestos et al., 2013). The phytochemicals may be responsible for anti-oxidant property of *R. communis* extract and showed significant activity by different methods.

The anti-oxidant activity in *R. communis* extracts using DPPH and nitric oxide scavenging assay correlates with the present study. The IC_{50} values of n-butanol and chloroform extracts by DPPH assay were found to be 140 ± 0.19 and 48560 ± 0.81 µg/mL, respectively. The IC_{50} values by nitric oxide assay of n-hexane, chloroform, ethyl acetate, and n-butanol extracts of *R. communis* was found to be 173.45 ± 0.84, 231.36 ± 0.91 and 109.77 ± 0.66 µg/mL, respectively (Iqbal et al., 2012).

Cytotoxicity was also showed by different plant extracts in human cell lines (Prakash and Gupta, 2013). The present study showed all the extracts possess cytotoxic activity against all the cancer cell lines used as compared to the normal cells, where no changes were observed. The methanolic plant extracts showed more activity and changes as compared to other extracts. The ethanolic extract of seed of *R. communis* showed 41% cytotoxicity against Colon 502713 cell lines, whereas the extract of stem showed 47% activity was against SiHa cell line using SRB assay (Prakash and Gupta, 2014). The extracts of *R. communis* leaves showed cytotoxicity against several human tumor cell lines having IC_{50} values ranging between 10-40 µg/mL and also showed apoptosis in SK-MEL-28 human melanoma cells (Darmanin et al., 2009). The *R. communis* leaf extract showed cytotoxic effect on A375 cell line with IC_{50} 48 µg/mL in concentration ranging between 25 to 100 µg/mL by MTT assay (Shah et al., 2015). The cytotoxic effect and anti-inflammatory activity of *R. communis* leaves extract showed percentage free radical ($ABTS^{+}$) scavenging activity of methanol 95%, acetone 91%, dichloromethane 62%, and hexane 50% at 2.50 mg/mL. The methanol extract had LC_{50} value of 784 µg/mL after 24-h exposure on Bud-8 cell line, whereas 629.3, 573.6 and 544.6 µg/mL in hexane, dichloromethane and acetone extract respectively (Nemudzivhadi and Masoko, 2014).

Conclusion

The methanollic extracts of *R. communis* in the present

study, were found to have the maximum activity and can be used as a therapeutic agent for curing number of microbial and cancers due to its anti-oxidant property. Therefore, all the extracts of *R. communis* studied were found to possess significant anti-bacterial, anti-oxidant and anti-carcinogenic activity. Further studies would be carried out for purification and characterization of the compounds.

Conflict of interests

The authors have not declared any conflict of interests

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