NUTRITIONAL COMPOSITION, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF CANARY MELON SEED (*Cucumismelo*) OIL

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Abstract: The chemical analysis revealed that canary melon seed (*Cucumismelo*) oil extracted using the solvent extraction method has saponification values, unsaponifiable matter content, acid value, peroxide values, free fatty acids, iodine, refractive index and specific gravity (SG) of 190.1 mg KOH g^{-1}, 4.1 g/kg^{-1}, 2.99 mg KOH g^{-1}, 6.92 meq O_2 kg^{-1}, 2.56%, 109.63 Wij's, 1.445 and 0.94, respectively. The percentage oil yield was 50.29% and oil was liquid at room temperature 30°C. Physical and sensory properties of canary oil showed the extracted canary melon seed oil was cream in colour, clear without impurities, light textured with fruity smell. Antimicrobial activity of canary melon seed oil was determined using the agar well diffusion method. Antibacterial and antifungal activity test of canary melon seed oil on twelve (12) clinical isolates showed that all the tested strains were susceptible to the oil. The investigation further revealed that the seed oil exhibited inhibitory effect on the growth of gram positive and gram negative clinical isolates tested. The seed oil showed moderate antibacterial activity with the average zone of inhibition of 9-12 mm. *Streptococcus pyogenes* (14 mm), *Staphylococcus aureus* (15 mm), *Pseudomonas aeruginosa* (13 mm*)* and *Escherichia coli* (16 mm) was strongly inhibited. Canary melon seed oil was also found to have strong inhibitory activity against the growth of *Aspergillusflavus* (17 mm) *and Candida albicans (*18 mm). The results indicated that canary melon seed oil has potential for nutritional and antimicrobial purposes.

Keywords: Canary seed oil, chemical analysis, antibacterial, antifungal

Introduction

One of the main accomplishments of modern medicine is the development of antimicrobial agents. In recent times, the development of drug resistance in human and plant pathogen has developed due to indiscriminate use, incessant and misuse or overuse of antimicrobial drugs used in the treatment of infectious diseases (Bello *et al*., 2013; Igara *et al*., 2017). The adverse side reactions and limited life span of antibiotics has necessitated the urgent search for natural antimicrobials from various sources such as medicinal or herbal plants. In folk medicine, plants are well-known natural sources for the treatment of infectious diseases since ancient times. This has urged microbiologists around the world to search for formulations of new natural antimicrobials and evaluate the efficacy of natural plant products as a substitute for antibiotics which are produced from chemical agents (Sen and Batra, 2012; Mujeeb *et al*., 2014). Since time immemorial plants have served as sources of drugs and pharmaceuticals for both man and animals. There are a millions of plants growing on earth that is underutilized, many of which possess therapeutic and pharmaceutical properties (Mazid *et al*., 2012). In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from plants (Bello *et al*., 2013; Tiwari *et al*., 2013; Igara *et al*., 2017).

Canary melon (*Cucumismelo L*) belongs to the botanical family *Cucurbitaceae* (Raji and Orelaja, 2014; Zeb, 2016). The *Canary melon* otherwise known as *golden* melon is in the inodorus cultivar group and it belongs to the *Cucurbitaceae* family alongside other well-known cucurbits such as water melon (*Citulluslanatus*), cucumber (*Cucumeropsismannii*) and pumpkin (*Telfairiaoccidentalis*) (Raji and Orelaja, 2014; Warra and Sheshi, 2015). *Cucurbitaceae* is an important family comprising of genetically diverse food plants. Majority of the plants in this family are sensitive to drought and frost (Petkova and Antova, 2015). Some important Cucurbit family members are pumpkin, gourd, cucumber, squash, crenshaws and water melon (Selale *et al*., 2012).

Canary melon has an extensive rooting system, an aerial stem, simple leaf, trailing and creeping habit (Ajuru and Okoli, 2013). The vine of the plant can creep or trail, and it produces pretty yellow flowers in the spring. The melon's mature size is up to 2 feet tall, and 10 feet long (Ajuru and Okoli, 2013; Petkova and Antova, 2015). The melon plant is native to central Asia including Japan and South Korea and its many cultivated varieties are widely grown warm regions world-wide. Canary melons are beautiful bright yellow hybrid melons. The flesh of this melon is pale green in color, with a soft texture when ripe. Ripe canary melons are large, bright yellow, oval–shaped fruit with a smooth skin, pale green to white inner flesh. At this stage, the texture of the flesh is notably succulent, almost wet and semi firm, similar to a ripe pear and cantaloupes (Ajuru and Okoli, 2013; Raji and Orelaja, 2014; Warra and Sheshi, 2015).

Canary melons are seeded and grown for its edible fruits. The seeds are flattened and may be cream or light yellow in colour (Raji and Orelaja, 2014). The fleshy portion is usually eaten fresh by itself for breakfast, lunch or dessert or as part of other dishes like soups, salads, smoothies and more (MallekAyadi *et al*., 2018). After eating, the remaining portion of the fruit especially the seed is often discarded as agrowaste. Although the seeds can be utilized for other food applications such as preservative, animal feed and oil extraction, contributing to less waste disposal and value addition. Canary seedsare potential oil sources and this has been reviewed by researchers who reported that the dehulled seeds contain approximately 60 % of oil (Raji and Orelaja, 2014; Warra and Sheshi, 2015). The high amount of oil, showing promising useful characteristics such as good colour, aroma and appearance makes these seeds suitable for industrial and medicinal applications (Warra and Sheshi, 2015; Selale *et al*., 2012).

The honey dew aroma of the melon fruit is due to the presence of (Z,Z)-3,6-nonadien-1-ol and phenylethyl alcohol (Ajuru

and Okoli, 2013). Canary melon has been implicated by so many researchers to possess high nutritional and medicinal value (Petkova and Antova, 2015; MallekAyadi *et al*., 2018). A few research studies have showed that canary melon contain high amount of oil and is a major sources of protein, carbohydrate, fat and oils and minerals (Berdiyev *et al*., 2009). The research of Raji and Orelaja, (2014) showed that the fruits are rich in vitamins including riboflavin, A, C, folic acid and thiamine and other health promoting plant compounds. Their research showed that hexane extracted oil contains physiochemical properties. Recently the work of Zeb (2016) has also shown that the fruits contain phenolics and possess antioxidant activities.

Various works on this plant were basically focused on the seed (Berdiyev *et al*., 2009; Raji and Orelaja, 2014; MallekAyadi *et al*., 2018). However, there are no reports on the antimicrobial properties of oil from canary melon. Therefore, the main objective of this study was to evaluate the physical, sensory and oil characteristics of canary melon seeds as potential source of valuable oil for nutritional and medicinal applications.

Materials and Methods

Materials

Collection and identification of plant

Fresh healthy fruits of canary melon were bought from fruit vendors at Sabo market, Yaba, Lagos State in May through June 2017. The fruits were taken to the plant science and Biotechnology laboratory, University of Salford, Manchester, United Kingdom where they were authenticated by a taxonomist and observed for spoilages.

Sample preparation and processing

Canary melon seeds were sorted, and damaged ones discarded. The selected healthy melons were washed in cold tap water to remove dirt adhering to the surface. After washing, the fruits were separated or cut into halves using a sharp knife and the seeds were removed. The seeds were then dried in the laboratory under room temperature for 14 days (two weeks). Thereafter, the seeds were milled using MarlexExcella mixer/grinding machine (Amazon, UK), packed in air tight containers and kept in the refrigerator at 4°C for further processing.

Methods

Extraction of canary melon oil

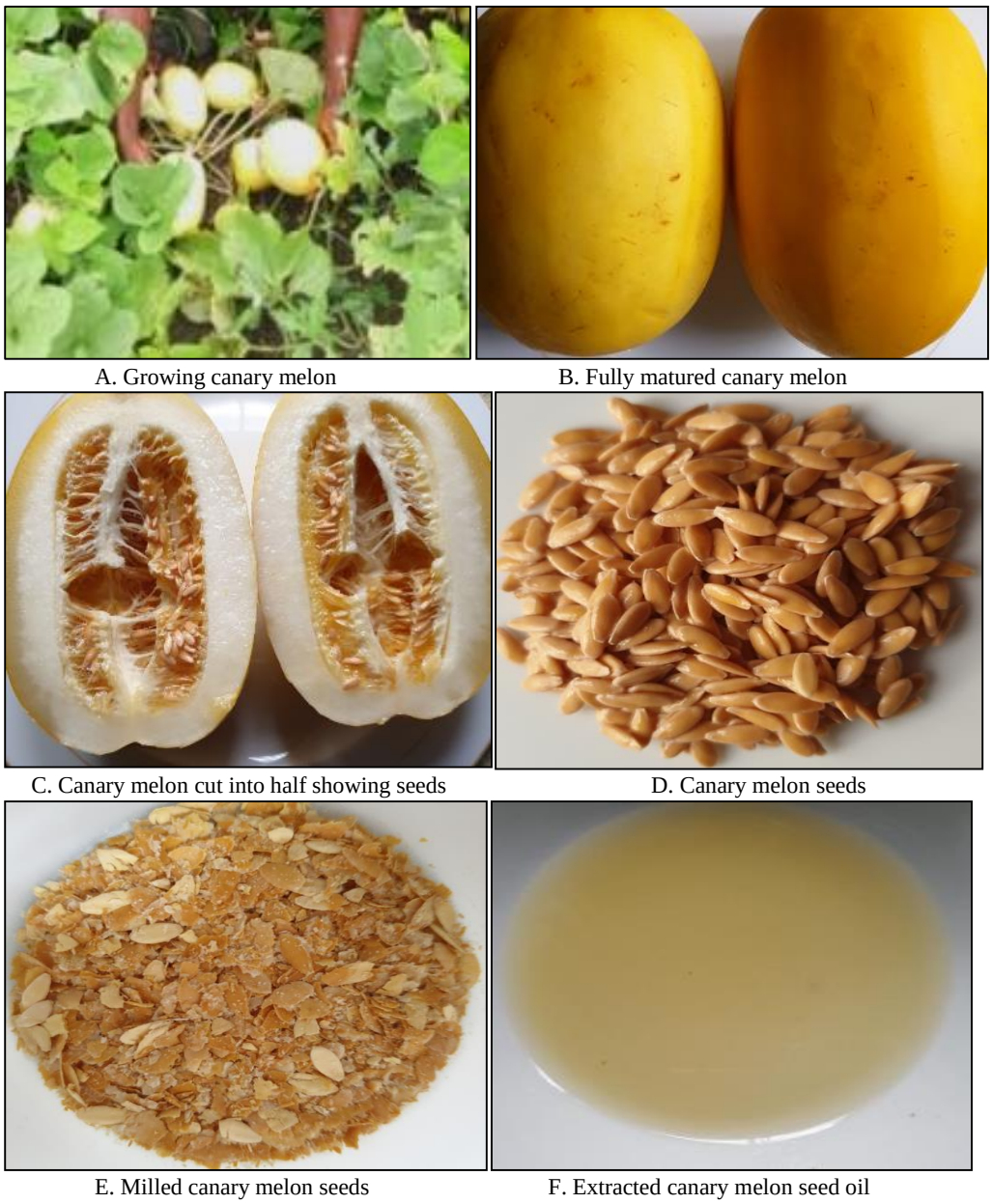
Oil was extracted from the dried grounded seeds of canary melon with n-hexane in a process known as solvent extraction. The method of Efeovbokhan *et al*. (2015) was employed for the extraction of oil from canary melon seeds. Prior to extraction, the pulverized seed samples were kept in an oven at 105ºC for 1 h to remove any moisture that may still be present. Fifty (50 g) of the dried seed sample was wrapped in a white muslin cloth and put into a porous thimble of the soxhlet extractor. Then, 250 ml of n-hexane of HPLC grade with boiling range of 60ºC was added. The soxhlet coupled with a condenser and flask already filled with the set up was heated in a heating mantle at 65°C to allow solvent boiling. In the process the solvent vapour travels up a distillation arm and flowed into the chamber housing the sample material. The extract seeps through the pores of the thimble and fills the siphon tube where it flows back down into the round bottom flask. The process was allowed to continue for 8 h until a clear solvent was obtained in the thimble chamber. At the end of the extraction, the resulting mixture of the oil was filtered with a 10 mm Syringe-driven with a filter 0.45 um to remove any impurities. The solvents were further removed completely with a rotary-evaporator (Model N-1, Eyela, Tokyo Rikakikal Co., Ltd., Japan). The extracted oil was stored in white bottles and tubes under nitrogen at 4°C until analyzed. After extraction, the physical and sensory properties of the seed oil were recorded, and the amount of oil obtained was measured using an analytical balance and result obtained was used to calculate the percentage yield of the oil sample.

Physical and sensory properties

The colour, texture, sedimentation, smell/aroma and other related physical characteristics of canary melon oil extracted were noted. A 10 man panel consisting of students of University of Salford, Manchester, United Kingdom was constituted to evaluate the colour, texture, sedimentation property and aroma of the extracted oil. Colour measurement was done with a Lovibond colorimeter.

Chemical properties

Chemical analysis was carried out on canary melon seed oil to determine the saponification values, unsaponifiable matter content, acid value, peroxide values, free fatty acids (FFA), iodine, Refractive index (RI) and Specific gravity (SG). The procedures of Egan *et al*. (1981) and AOAC (2000) were adopted for the estimation of the oil sample. The saponification value was determined by the estimation method. About 100 mg of the extracted oil was mixed with 0.5 M ethanolic potassium hydroxide solution and boiled under reflux on a water bath for 30 minutes. The solution was then titrated with 0.5 M HCl, using phenolphthalein solution as indicator. The saponification value was calculated using the titration values.For the determination of unsaponifiable matter, about 100 mg of the extracted oil was heated under reflux and saponified with 5 mL of ethanolic potassium hydroxide solution (20% w/v) for 2 h. The unsaponifiable matter was extracted thrice with 15 mL of petroleum ether and the extracts were combined and evaporated at 40°C under reduced pressure. The unsaponifiable residue was weighed. For peroxide determination, a known weight of canary melon oil was dissolved in a mixture of acetic acid/chloroform (3:2 v/v) and a saturated solution of KI (1 mL) was added. The liberated iodine was thereafter titrated with sodium thiosulphate solution (0.05 m) in the presence of starch as indicator. For the free oil acidity (acid value), the titration method was used. For this determination, a known weight of canary melon oil was dissolved in a mixture of ethanol (95 %) and ether, previously neutralized with 0.1 M potassium hydroxide solution to phenolphthalein solution. The mixture was then titrated with 0.1 M potassium hydroxide solution as indicator until the solution remained faintly pink after shaking for 30 seconds. Free fatty acid (FFA) determination was by colorimetric method, while iodine value was determined using Wij's (iodine monochloride) method. In this method, a weighed amount of oil sample was dissolved in carbon tetrachloride and added to iodine monochloride. The resulting solution was kept in the dark for 30 min and then titrated with 0.1 M sodium thiosulphate, using starch solution as the indicator.Refractive index (RI) was measured with a Refractometer (RFM342, Bellingham + Stanley, England) while the specific gravity (SG) of the oil sample was determined gravimetrically. Both were determined using the methods of Egan *et al*. (1981).



A. Growing canary melon B. Fully matured canary melon

C. Canary melon cut into half showing seeds D. Canary melon seeds

E. Milled canary melon seeds F. Extracted canary melon seed oil

Fig. 1: Shows the flow chart for the extraction of oil from canary melon seeds

A = Growing canary melon. B = Fully matured canary melon seeds. C = Mature canary melon cut into half showing seeds. D = Canary melon seeds. E = Milled canary melon seeds. F = Extracted canary melon oil

Test organisms

All the strains of bacteria and fungi species used in this study were clinical isolates. The bacterial and fungal species were obtained from the Department of Microbiology Laboratory, University of Salford, Manchester, United Kingdom.

Gram positive bacteria

Streptococcus faecalis, Streptococcus pyogenes, Enterococcus faecalis, Bacillus subtilis and *Staphylococcus aureus.*

Gram negative bacteria

Serratiamarcescens, Klebsiellapneumoniae, Proteus vulgaris, Pseudomonas aeruginosa and *Escherichia coli*.

Fungi

Aspergillusflavus and *Candida albicans*.

Re-identification of the organisms

Identification of all the isolates in this study was done in accordance with the technique of Cheesbrough (2010). The identities of the isolates were however reconfirmed using standard morphological, biochemical methods and mycological diagnostic methods. All the bacterial test organisms were aseptically grown on 5 ml nutrient broth overnight at 37ºC then subcultured onto MacConkey agar (MCA) and nutrient agar (NA) medium plates to get pure cultures of the isolates. These plates were thereafter incubated at 37ºC for 24 h. Fungal strains were cultured on Sabouraud's Dextrose Agar (SDA) plate. Cultured plates were incubated at 25°C for 48 h. Pure cultures of these isolates were identified biochemically using standard microbiological identification techniques described by Cheesbrough (2010).

Microbiological screening

Preparation of culture media and its sterilization

Microbial culture media including nutrient agar (NA) and nutrient broth (NB) were purchased from Oxoid, UK. Mueller Hinton Broth (MHB) and Mueller Hinton agar (MHA) were obtained from Sigma Aldrich, Dorset, UK. All these media were suitable for the growth of bacteria cultures. For fungi culture, Sabouraud's Dextrose broth (SDB) Sabouraud's

Dextrose Agar (SDA) was obtained from Thermo Scientific, Hampshire, UK. All media used were prepared according to manufacturer's instruction. The media for both bacterial and fungal cultures were made up in large volumes as follows: NA, (28 g), NB, (13 g), MHB (23 g), MHA (38 g), SDB (13 g) and SDA (65 g) were separately made up in 1 liter (1000 ml) of deionized water and sterilized at 121ºC for 15 min in an autoclave and subsequently allowed to cool after sterilization to about 45ºC (temperature at which the agars remains molten) before pouring into petri dishes to solidify.

Preparation and standardization of inoculums for antibacterial and antifungal activity

To investigate the antibacterial activity of the extracted canary melon seed oil, inoculums for agar well diffusion tests were prepared in accordance with the guidelines of Clinical Laboratory Standards Institute, CLSI (formerly the National Committee for Clinical Laboratory Standards) (CLSI, 2013) and SenandBatra (2012). Using aseptic techniques, the bacteria strains were cultured separately in 10 ml of nutrient broth and Mueller Hinton Broth overnight or to stationary phase (OD600 = >2.5). From the overnight culture, using streak plate method, a loop full of the tested strains were streaked across the respective agar plates and incubated at 37ºC for 24 h. From the incubated plates, inoculums were prepared by making a direct broth suspension of four to five well isolated colonies of the Gram positive and Gram negative bacterial strains with the same morphological type in freshly prepared 10 ml broths in separate test tubes and incubated in a shaking incubator (Camlab, UK) at 37ºC for 18 h with 200 rpm. The bacterial suspensions were thereafter adjusted 0.5 McFarland standards (equivalent to 1.5 $x10^8$ CFU/ml). This was done by diluting the 18 h bacterial cultures 1:100 with respective sterile broths before growing them back in the shaking incubator for approximately 2-3 h (mid-log phase) to obtain 0.08 to 0.10 OD625 corresponding to 1.5 x 10^8 CFU/ml. The correct density of the turbidity standard was verified using a spectrophotometer with a 1 cm light path and matched cuvette (Star Labs, UK) to determine the absorbance at 625 nm. Blank of MHB alone was used to calibrate the spectrophotometer before measuring the samples. To ensure conformity of the suspension's turbidity with McFarland standard, both the suspensions and the prepared McFarland standard were also compared visually. Furthermore, the inoculums suspension was used within 30 min of standardization, which is a very important factor to avoid any change of the size of inoculums or loss of their viability.

Forthe antifungal activity of the canary melon seed oil, the fungal suspensions used was prepared using three to four morphologically similar colonies of the fungal strainsfrom a 24 h culture on Sabouraud dextrose agar. The turbidity of the fungal suspension was adjusted to 1.0 McFarland standard (equivalent to 1.5 × 10^8 CFU/ml) with sterile normal saline (0.89% NaClwt/vol). All experiments were performed in duplicate and repeated three times.

Determination of antibacterial and antifungal activity of canary melon seed oil

Agar well diffusion assay

Antimicrobial activity of canary melon seed oil was tested against Gram positive, Gram-negative bacteria and fungal strains using Agar well Diffusion method described by Perez *et al*. (1990) and Adeniyi *et al*., (1996). In this method, nutrient agar and Mueller Hinton agar (MHA) plates were seeded with 0.2 mL of 18 h broth cultures of each bacterial isolate. For the Fungal isolates Sabouraud's Dextrose Agar (SDA) was seeded with 0.2 mL of 24 h broth cultures offungal strains. Thenutrient agar, Mueller Hinton agar (MHA) and Sabouraud's Dextrose Agar (SDA) were separately seeded byspreading a small volume (0.2 mL) of the liquid inoculums (sub-cultured broth media of both bacteria and fungi isolate) by means of an L-shaped glass rod (or a "spreader") in such a way that the surface of the agar in the plates were covered with the microbes (test organisms). All test microorganisms were separately seeded into different plates. Allthe plates were left to dry for 1 h. Asterile 6 mm cork-borer was used to cut two wells of equidistance in each of the plates and 0.2 mL (200 µl) of the seed oil was introduced into one of the two wells while the same amount of sterile oil was introduced into the second well as control (used as negative control) and all the plates were aerobically incubated at 37ºC for 24 h for the bacteria and 48 h for the fungi. The diameter of zones of inhibition was measured by means of linear instrument in millimeter (veniercalliper) and recorded.

Results and Discussion

The physical and sensory analysis of the colour, texture, sedimentation properties, smell/aroma and state of canary melon seed oil indicated that it was cream in colour, light, clear without impurities with fruity smell. The oil is liquid at room temperature 30°C (Table 1).

Table 1: Physical and sensory attributes of canary melon seed oil

Attributes	Characteristics / Properties
Colour	Cream, clear and transparent ($5^1/_4$) on Lovibond meter)
Texture	Light
Sediments	Free of sediments. No impurities
Smell/aroma	Fruity smell coming from the plant material.
State at room temperature	Liquid.

Source: Laboratory analysis (2017).

Table 2: Chemical composition of canary melon seed oil

Quality parameters	Values
Oil yield	50.29 %
Specific gravity	0.94±0.01
Refractive index	1.445±0.002
Acid value (mg KOH/g)	2.99±0.02
Free fatty acid (%)	2.56±0.02
Peroxide value (meq O_2/ kg^{-1})	6.92±0.02
Saponification number (mg KOH/g)	190.1±0.03
Unsaponifiable fraction (g/kg^{-1})	4.1±0.01
Iodine (mg of $1g^{-1}$ of oil) (Wiji's)	109.63±0.03

Each data is mean of three replicate readings ± Standard Deviation (SD)

Source: Laboratory analysis (2017).

The chemical analysis of the oil from canary melon seed are shown in Table 2. The data (Table 2) shows the saponification values, unsaponifiable matter content, acid value, peroxide values, free fatty acids, iodine, refractive index and specific gravity (SG) of the oil was 190.1 mg KOH g^{-1}, 4.1 g/kg^{-1}, 2.99 mg KOH g^{-1}, 6.92 meq O2 kg^{-1}, 2.56%, 109.63 Wij's, 1.445 and 0.94. The oil yield from canary melon seed was 50.29%. The refractive index and specific gravity values of 1.445 and 0.94 compares favorably well with that of African walnut oil (1.446 and 0.97) as reported by Ozcan (2009). Acid value of canary seed oil was 2.99 mg KOH g^{-1}, lower than those of soya bean (4.30 mg KOH g^{-1}) and turkey (5.61 mg KOH g^{-1}) reported by Obasi *et al*. (2012). This was within the range reported for watermelon (3.41 mg KOH g^{-1}) and melon seed (4.26 mg KOH g^{-1}) by Raji and Orelaja (2014). Free fatty acid value of oil sample was 2.56 %, FFA value is an important value in considering the keeping quality of oil

because the lower the FFA value, the better the quality of the oil when stored. The peroxide value is also lower than the generally recommended value for commercial edible crude vegetable oil. The peroxide value of 6.92 meq O_2 kg^{-1} obtained for the seed oil is lower than 19.54 meq O_2 kg^{-1} reported by Ebechi and Avwobobe (2006) for melon seed oil. The value obtained appears lower than the codex value standard for both refined (10.0 meq O_2 kg^{-1}) and unrefined oil (20.0 meq O_2 kg^{-1}) (WHO, 1993). This indicates that canary oil has lower degree of rancidity. This is also an indication that canary seed oil can resist lipolytic hydrolysis and oxidative deterioration (WHO, 2002). A high saponification value of 190.1 mg KOH g^{-1} indicates that the oil has low molecular weight fatty acids. This attribute is of importance in soap making as well as in shampoo making (Ajiwe, 1994; Akanni *et al*., 2005). The value obtained is the range of values (174.84 - 192.45 mg KOH g^{-1}) obtained for rapeseed oil and soyabean oil that have gained much market priority as reported by Obasi *et al*., (2012). The value obtained for canary melon seed oil is this study is higher than those of watermelon (175.98 mg KOH g^{-1}) and melon seed (201.15 mg KOH g^{-1}) reported by Raji and Orelaja (2014). This value is also in the range of values reported for *Cucurbitaceae* seed oil (182.1 – 193.8 mg KOH g^{-1}) (Mabaleha *et al*., 2007). Unsaponification value was found as 4.1 g/kg^{-1}, and similar to the value of 4.7 g/kg^{-1} reported for terebinth fruit by Ozcan, (2004). Obviously, this attribute of the oil can be linked with the cholesterol content it contained. Iodine value for the oil was 109.63 Wij's. The iodine value of canary oil is lower than those of sunflower (110-143), soybeans (120-143), rubber seed (134.51) (Abayeh *et al*., 1999) and (124.0) in desert melon but higher than those of castor oil (83.75) and *Coulaedulis* (90-95) (Adamu *et al*., 2013). This value which is in the middle range shows that canary oil is semidrying and unsaturated. This further shows that the oil could be used in liquid soap formulation, just as the low acid value recorded for the oil could be of significance in paint and varnish manufacturing (Popoola and Yangomodou, 2006).

Antibacterial and antifungal activityof the oil from canary melon seed was tested on Gram positive; *Streptococcus faecalis*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Bacillus subtilis* and *Staphylococcus aureus*, Gram negative; *Serratiamarcescens*, *Klebsiellapneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Escherichia coli* and fungal isolates; *Aspergillusflavus* and *Candida albicans*.

Table 3: Antibacterial and antifungal activity of canary melon seed oil against microorganisms

Microorganisms	**100 % Canary melon seed oil**	**Sterile oil**
Bacterial strains (Gram positive strains)	**Zones of inhibition by seed oil (mm)**	
Streptococcus faecalis	12	-
Streptococcus pyogenes	14	-
Enterococcus faecalis	10	-
Bacillus subtilis	9	-
Staphylococcus aureus	15	-
(Gram negative strains)		
Serratiamarcescens	10	-
Klebsiellapneumoniae	10	-
Proteus vulgaris	11	-
Pseudomonas aeruginosa	13	-
Escherichia coli	16	-
Fungal strains		
Aspergillusflavus	17	-
Candida albicans	18	-

Source: Laboratory analysis (2017).

The result of the antibacterial and antifungal activity of canary melon seed oil is presented in Table 3. The antibacterial and antifungal activity was determined by measuring the diameter of zones of inhibition. The result of this investigation showed that the seed oil exhibited inhibitory effect on the growth of gram positive and gram negative clinical isolates tested. The seed oil showed moderate antibacterial activity with the average zone of inhibition of 9-12 mm. *Streptococcus pyogenes* (14 mm), *Staphylococcus aureus* (15 mm), *Pseudomonas aeruginosa* (13 mm) and *Escherichia coli* (16 mm) was strongly inhibited. Canary seed oil was found to have strong inhibitory activity against the growth of *Aspergillusflavus* (17 mm) and *Candida albicans* (18 mm). No growth was observed in the control sterile oil.

Conclusions

Canary melon seed oil demonstrated a broad spectrum activity on bacterial and fungal clinical isolates tested. The antimicrobial activity of the seed oil shows that it can be exploited for use in traditional medicine, pharmaceutical and cosmetic industries.

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Conflict of Interest

The authors declare that there is no conflict of interest reported in this work.

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