Journal of Food Research; Vol. 9, No. 2; 2020
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Evaluation of Five Essential Oils by Gas Chromatography-Mass Spectrometry and their Effect on Fungal Growth Inhibition and Sensory Acceptability of Soymilk

Marguerite Niyibituronsa[1, 2, 3], Arnold Nola Onyango[2], Svetlana Gaidashova[1], Samuel Imathiu[2], Zhang Ming[3], Yang Ruinan[3], Zhang Weiqi[3], Wang XiuPin[3], Zhang Qi[3], Zhang Zhaowei[3] & Li Peiwu[3]

[1]Rwanda Agriculture Board, Rwanda

[2]Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

[3]Oil Crops Research Institute of the Chinese Academy of Agriculture Science, Wuhan, China

Correspondence: Marguerite Niyibituronsa, Rwanda Agriculture Board, Rwanda, P.O.BOX 5016 Kigali, Rwanda. Tel: 250-788-848-200. E-mail: niyibituronsam@gmail.com

Received: December 29, 2019 Accepted: February 15, 2020 Online Published: March 17, 2020

doi:10.5539/jfr.v9n2p36 URL: https://doi.org/10.5539/jfr.v9n2p36

Abstract

Essential oils are widely used in the food industry as natural food preservatives to extend product shelf life and as flavoring agents. However, not much has been done on their use in soymilk. The aim of the study is to determine the compounds of five essential oils by GC-MS and their effect on fungal growth inhibition and sensory acceptability of soymilk. The components of the essential oils of five spices, namely citronella, basil, cinnamon, eucalyptus and mint were analysed by gas chromatography-mass spectrometry (GC-MS). The minimum inhibitory concentration (MIC) of the essential oils was tested on the fungus *Aspergillus flavus* 3.4408 on PDA (agar dilution method). Sensory evaluation of soymilk flavored with the essential oils of citronella, basil and mint at different concentrations was done by ten member panelists using a 9-point hedonic scale. The main compound for basil was eugenol 83.26%. Cinnamon contained cinnamaldehyde (97.3%). The main compounds in citronella (*Cymbopogon nardus*) were limonene (38.51%), citronellal (30.29%). Eucalyptus (*Eucalyptus globulus*) essential oil mainly contained eucalyptol/cineole (76.70%), and Mint (Mentha arvensis): Menthol 42.72%, Menthone 25.72%. The MICs of citronella, basil, cinnamon, eucalyptus and mint were 5-10 µl/ml, 0.5-1 µl/ml, ≤0.1 µl/ml, >>10 µl/ml and 10-20 µl/ml, respectively. Thus, cinnamon was the most effective in inhibiting fungal growth, while eucalyptus was the least effective. These essential oils improved the soymilk flavor. Mint was the most preferred flavor, followed by citronella and basil. Thus, essential oils especially mint and citronella can be used for improving acceptability of soymilk at low concentration.

Keywords: basil, CFU, citronella, cinnamon, eucalyptus, mint, sensory attributes, soybean

1. Introduction

Soybean contains oil with polyunsaturated fatty linoleic and linolenic acids and the oxidation of those fatty acids during processing is associated with the beany flavors responsible for reduced consumer acceptability of soybean milk (Davles, 1987). Apart from attempts at reduction of off flavours from soymilk during extraction, other options have been undertaken to remove the beany odor (Kinney, 2003; Hideo, 1979). For example, Davles (1987) carried a study to remove the off flavor by reducing lipoxygenase genetically to improve soybean products acceptability. Orange juice, pineapple and banana have also been used to improve the taste of soybean milk (Kale et al., 2012; Laswai et al., 2009). However, not much has been done on the use of essential oils for improving soybean flavor.

Essential oils are active botanical constituents derived from plant materials such as leaves (lemon grass, ocimum, mint), leaves and stems (geranium, patchouli, cinnamon), flowers (rose, mimosa, lavender), seeds (fennel, coriander, nutmeg), fruits (orange, lemon, bergamot), rhizomes (ginger, curcuma, orris), gums (storax, myrrh, balsam of Peru), bark (cinnamon, cassia, canella), roots (vetiver, valerian, angelica) and wood (cedar, sandal, pine) (Handa et al., 2008). Different methods are used to extract essential oils, namely, water steam distillation, Solvent Extraction, CO_2 Extraction, Maceration, Effleurage, Cold Press Extraction, and water and steam distillation. The last is the most favored for oil quality and higher yield (Handa et al., 2008). The boiling point of

essential oils, a mixture of monoterpenes, sesquiterpenes and their oxygenated products, ranges from 150 ℃ to 300 ℃ higher than the boiling point of water 100 ℃ but by heating and moisture from the steam the pressure increase in the plant material and the oils volatilize from the plant (Tandon, 2008). For better yield and quality of the essential oils the temperature should be maintained as low as possible and plant material packed well in the distillation still (Koul et al., 2004). Fresh oils do not have color but with time oxidation may occur, leading to dark color. Thus, they need to be stored in a cool, dry place in a dark glass container and close tightly (Rassem et al., 2016). The essential oils (EOs) are used in wide variety of food industry as food preservative (Hyldegaard et al., 2012). They have antibacterial and antifungal properties and are increasingly used as an alternative of synthetic products (Nazaro et al., 2017). The composition of essential oils of any species varies according to genotypes and environmental factors. For example, three different chemotypes were reported for the essential oil of *Ocimum ciliatum* accessions in Iran (Moghaddam et al., 2017). Therefore, in utilizing essential oils for improving the flavor of soymilk, it is necessary to characterize the chemical composition of the essential oils to allow for product standardization.

Basil essential oils are used to flavor foods (Ozcan and Chalchat, 2011). For flavoring recipes, 1-3 drops of basil essential oil is better than the use of fresh or dried basil (Sustainable Baby Steps, 2019). Mint can also be used as flavoring agent in food (Fatih et al., 2017). Essential oils from Cymbopogon (Citronella and Lemon grass), an aromatic tropical plant in the family of Poaceae, which gives flavor to recipes including tea, can potentially be used for improving soymilk flavor. Essential oil of Cymbopogon has been obtained by water steam distillation (Millet, 2015, Ranitha et al., 2014). Cymbopogon is relatively cheap and available (Laswai et al., 2009). However, these sources of essential oils have not been tested for quantity of extract to be used in soybean milk.

2. Materials and Methods

2.1 Essential Oils Compounds Analysis by GC-MS

Soybeans and essential oils (*Cymbopogon nardus, Ocimum basilicum, Cinnamomum verum, Eucalyptus globulus* and *Mentha)* were purchased at Hengcheng natural perfume oil co., Ltd, JiangXi province, China. Analysis of EOs compounds by Gas Chromatography- Mass Spectrometer (GC-MS) was done using GC-MS Agilent at OCRI; Mass spectra were obtained on Agilent 5973 MSD mass spectrometer, coupled directly to 7890A gas chromatograph fitted with a J & W DB-5ms, 0.25 mm i.d. x 30 m, 0.25 micron coating thickness, fused silica capillary column. The GC/MSD was operated under the following conditions: injector temperature - 240˚C; transfer line - 300˚C; the column temperature was initially held at 50˚C for 3 min, increased to 240˚C at a rate of 3˚C/min, and then held at this higher temperature for 2 min; injection - 0.1 µl (10% soln.), split 1:20, and helium with a flow rate of 1.2 ml/min was used as the carrier gas; Electron energy of 70 eV in the electron ionization mode, and an ion source temperature of 200˚C. Scan Range 41 - 415, 1 scan/ sec., Solvent delay 2.00 min (Adams, 2017). A comparison of retention time of compounds with the standards was done. In addition, a comparison of mass spectra of the components with the mass spectra stored in the National Institute of Standards and Technology (NIST) reference library was done, and the calculation of percentages of compounds in the EOs.

2.2 Test of EOs Minimum Inhibitory Concentration on Fungal Growth

Strains of fungi species *Aspergillus flavus* 3.4408 was obtained from the culture collection of Oil Crops Research Institute of the Chinese Academy of Agricultural Science, Department of Mycotoxins Research, Wuhan in China. The fungi were cultured in petri dishes on Potato dextrose agar (PDA) for 7 days at 28˚C. Extraction of the spores was done by washing the colonies in petri dishes with Tween 80 0.1% v/v distilled water. Using a pipette tip, the extract/spores suspension was collected in a tube (Moosavi-Nasab et al., 2018). The suspension was tested for contamination by adding 0.5 ml in 50ml of Liquid Sabourand Medium and incubating at 28 ℃ in an incubator shaker for 2 days. If not contaminated hyphens growth was visible in small ball in the medium. The spore concentration was determined using a hemocytometer slide by an optical microscope (Nikon eclipse E100, Japan) and the *Aspergillus flavus* spore suspension was diluted in 0.1 % Tween 80 to bring the final inoculum to 5.10^5 CFU/ml (CLSI, 2012).

The EOs and Medium PDA were prepared using agar dilution method (Hammer et al., 1999; Davari and Ezazi, 2017). The concentrations of 1, 3, 5, 10, 20, 50 and 100 µl /10mL of PDA were used. Inoculation was done with 10 µl of fungal suspension in triplicate and sample control. Petri dishes were sealed and incubated at 28 ℃. The growth was observed on 2 days basis and each fungal colony was measured in two perpendicular directions, and the colony mean diameter was obtained after 7 days. The minimum inhibition concentration (MIC) was determined using the formula: Inhibition % = (C - T/C)*100; Where C: Diameter of fungal colony in control plates and T: Diameter of fungal colony in treated plates (Davari and Ezazi, 2017; Li et al., 2016).

2.3 Sensory Evaluation of Soymilk Flavored with EOs

Different levels of flavor one drop, 2 drops and 3 drops were added in 1L of soymilk except the control. The method of soymilk preparation that preserves nutrient was used (Hosken, 1999; Niyibituronsa et al., 2019; Nyagaya, 2008). A taste panel of ten members determined the acceptability of different soymilk flavored with Basil, Cymbopogon and Mint by using a 9-point hedonic scale from like extremely = 9 to dislike extremely = 1 (Hashmi, 2007). The products were evaluated for the taste, color, appearance, odor using an evaluation form (Appendix 1).

2.4 Data Analysis

Data was analyzed using SPSS 22.0 and MS Excel. Analysis of variance (ANOVA) was conducted and significance of differences between samples was declared significant at $P< 0.05$ probability levels.

3. Results and Discussion

3.1 Analysis of Essential Oils Compounds by GC-MS

The five essential oils were analysed for compounds by GC-MS. Figure 1 shows the compounds in Basil (*Ocimum basilicum)* essential oil. The main compounds for Basil were eugenol (83.26% at 22.06 Retention time (RT)) and Caryophyllene (10.36% at 23.98 RT).

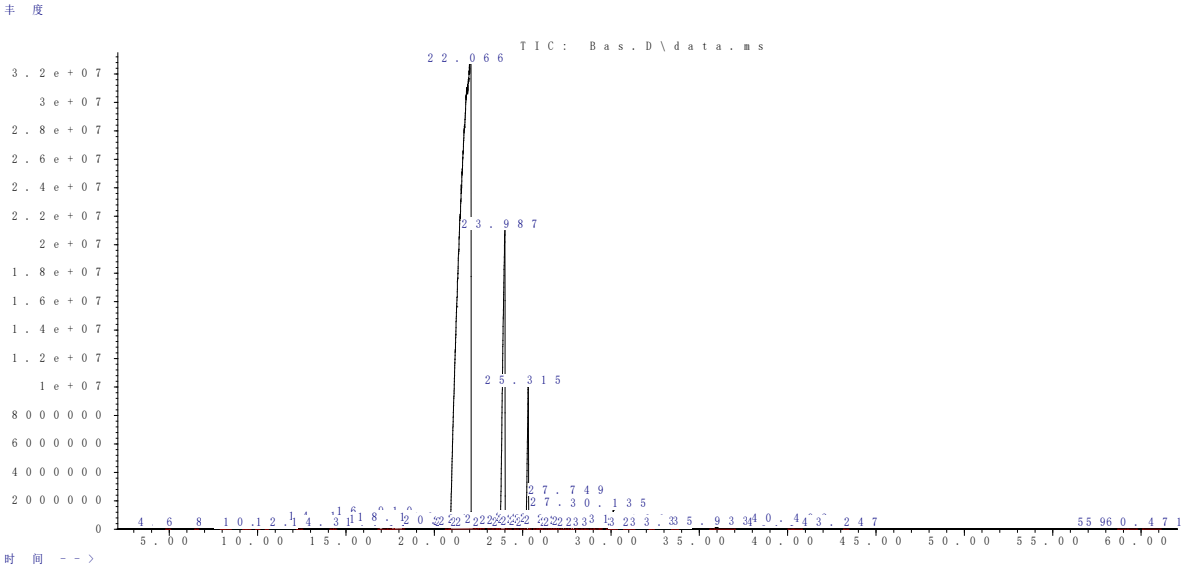

Figure 1. Basil essential oil compounds

Ocimum genus counts over 150 species. Depending on the species, growth stage, season and the geographic area where the plants are found, the compounds vary (Chamorro et al., 2012; Poonkodi, 2016). Some have eugenol as major compound up to 87%, especially for species of eugenol chemotype, similar to our findings (Koutsos et al., 2009; Muráriková et al., 2017). Others have linalool and chavicol as major compounds (Chamorro et al., 2012; Muráriková et al., 2017; Poonkodi, 2016). According to De Martino study, the main constituents of basil oil were *iso*-pinocamphone (35.10%) and carvone (39.70%), while eugenol was in trace, only 1% (De Martino et al., 2009).

The compounds for Cinnamon (*Cinnamomum verum*) are shown in Figure 2. The main compound is Cinnamaldehyde (97.26% at 18.99 RT).

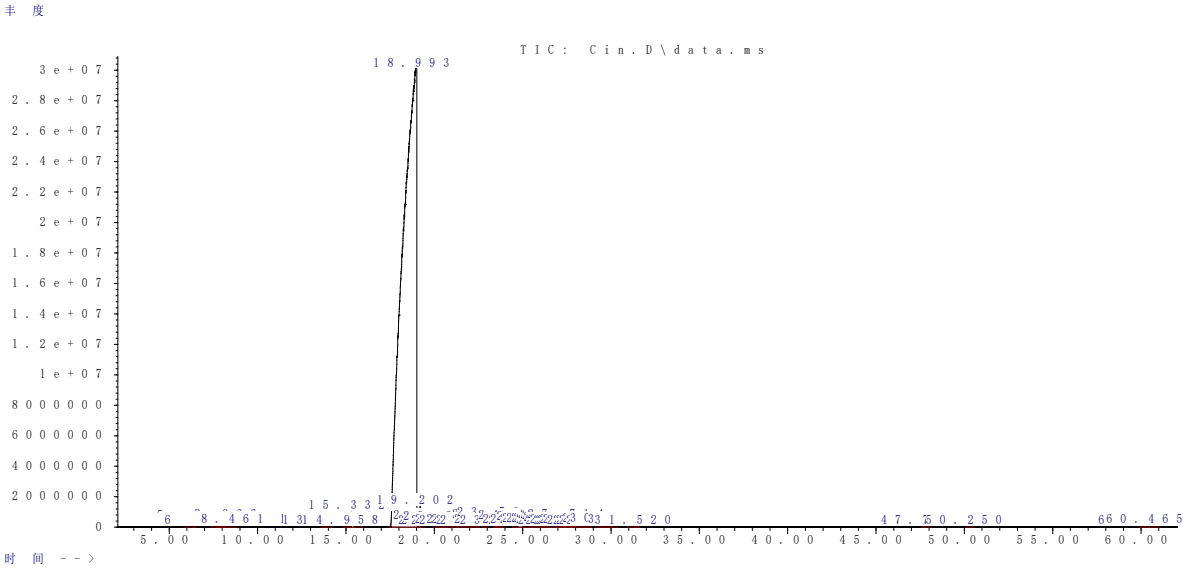

Figure 2. Cinnamon essential oil compounds

The composition depends on the growth stage and the segment of the plant (Vangalapati et al., 2012). Cinnamon leaves essential oil contains cinnamaldehyde 1.00 to 5.00% and eugenol: 70.00 to 95.00% while the bark contain cinnamaldehyde 65.00 to 80.00% and eugenol 5.00 to 10.00% (Jayaprakasha et al., 2002; Rao and Gan, 2014).

The compounds in Citronella (*Cymbopogon nardus*) are given in Figure 3 and the main compounds are Limonene (38.51% at 8.23 RT), Citronellal (30.29% at 8.17 RT), Citronillol (14.32% at 12.84 RT), Geraniol/citral (7.9% at 14.61 RT), and Cuparene (9% at 17.02 RT).

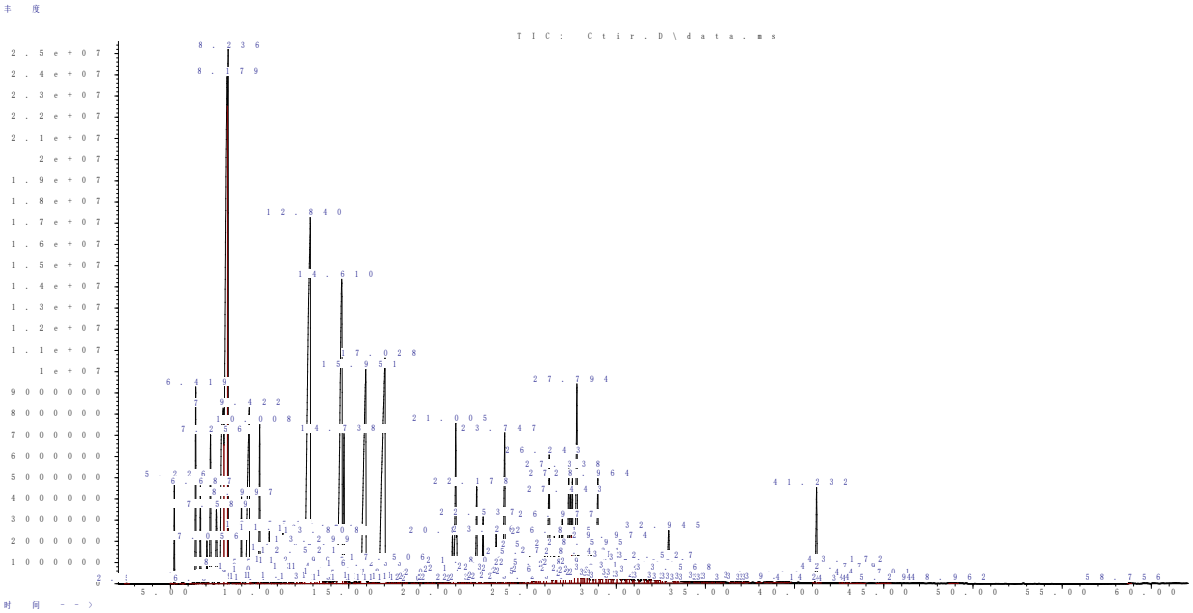

Figure 3. Citronella essential oil compounds

Another study reported Citronellal (33.8%), geraniol (21.6%), citronellol (9.2%)(Regnault-Roger, 1997). Compounds differ from one species of Cymbopogon to another; *C. martini* has Geraniol (64.0%-92.6%), *C. Flexuosus* Citral (80.6%-84.4%), *C. pendulus (*Lemongrass*)* Citral (75.9%), limonene (5.5%), *C. winterianus* of Kashmir Citronellal (31.1-35.4%), Geraniol (22.4-30.2%), Citronellol (7.4- 11.0%) while *C. Winterianus* of Himalayan region of India contain Geraniol (50.1%), Citral (21.8%), Citronellal (11.8%) (Wany et al., 2013).

Figure 4 shows compounds in Eucalyptus (*Eucalyptus globulus*) and the main compounds are Eucayptol/Cineole (76.70% at 8.48 RT), Carene (9.70% at 7.61 RT), Terpinene (4.16% at 6.71 RT) and Phellandrene (3% at 7.26 RT).

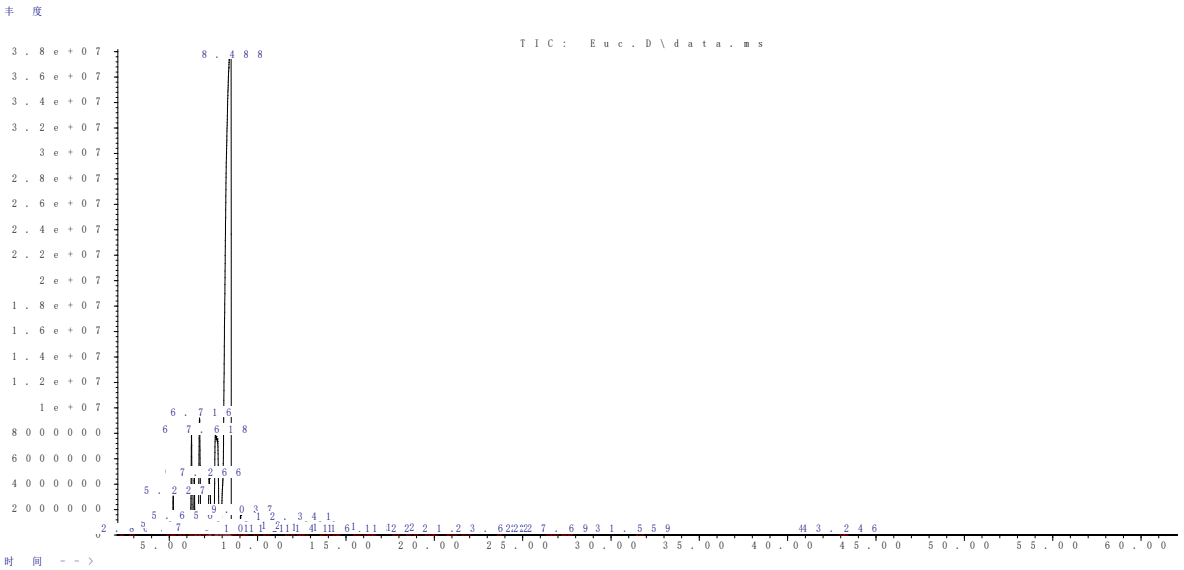

Figure 4. Eucalyptus essential oil compounds

Previous study reported cineole content of eucalyptus essential oil as 86%, pinene 3.9% and cymene 2.4% (Regnault-Roger, 1997). Some study found 44.08% of Cineole, 1.51% of Terpinene and many traces of compounds (Davari and Ezazi, 2017). Chemical compounds may vary within the same species due to the development stage of the plant used to extract the essential oils or the plant adaptation to the environment (Chamorro et al., 2012).

In mint (mentha) essential oil, the compounds are shown in Figure 5 and the main compounds were Menthol (42.72% at 14.25 RT), Menthone (25.72% at 13.04 RT), Limonene (4.92% at 8.05 RT) and Pinene (3.18% at 5.25 RT).

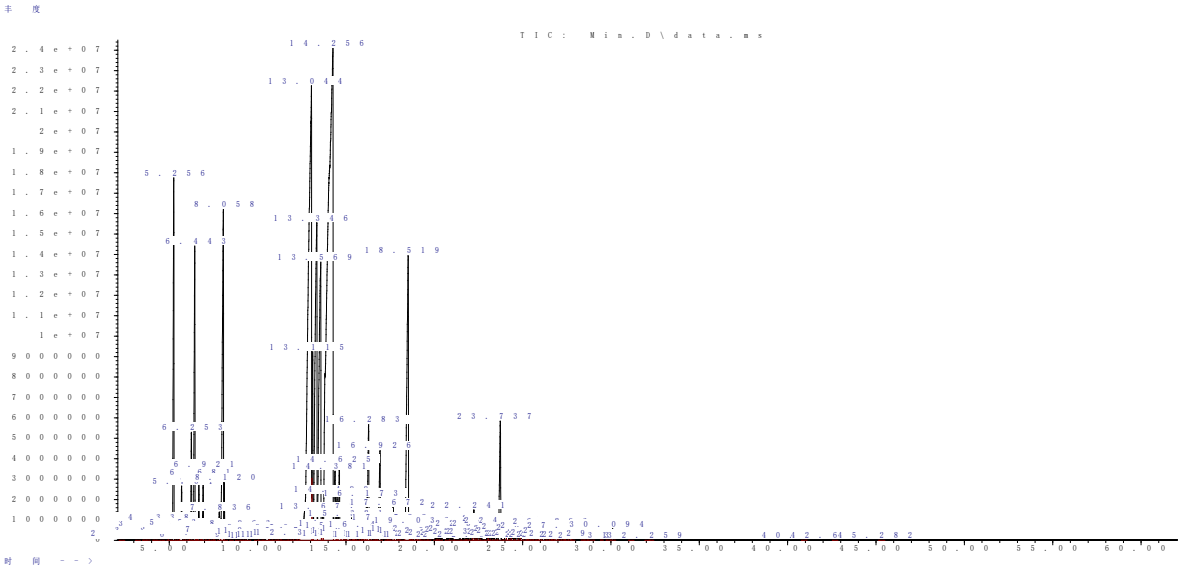

Figure 5. Mint essential oil compounds

Previous studies found menthol 85.89%, menthone 2.99% (Lopez-Reyes et al., 2010); Pino et al. (1999) found the major constituents as menthol (51.68%), menthone (26.08%) and menthyl acetate (10.55%) (Pino et al.,

1996).

3.2 Essential Oils on Fungal Growth Inhibition

The Minimum Inhibitory Concentrations MICs were determined as the lowest concentration of essential oil inhibiting the visible growth of each organism on the Potato Dextrose Agar PDA plate, Table 1. The MIC was estimated at one fold the concentration > 80% of inhibition (CLSI, 2012; Carson et al., 1995).

Table 1. The minimum inhibitory concentration of EOs on fungal growth

Essential oil	Scientific name	$MIC_{>80\%}$ (µl/ml)
Cinnamon	*Cinnamomum verum*	≤0.1
Basil	*Ocimum basilicum*	0.5-1
Citronella	*Cymbopogon nardus*	5-10
Mint	*Mentha arvensis*	10-20
Eucalyptus	*Eucalyptus globules*	>>10

Cinnamon is more effective for fungal growth inhibition at the concentration less than 0.1 µl/ml followed by Basil at 0.5 µl/ml and Citronella 5 µl/ml. Mint showed fungal inhibition growth at 10 µl/ml, the limit concentration set for the study and Eucalyptus didn't inhibit the fungal growth, it required higher concentration than 10 µl/ml.

In Figure 6 the concentration of EOs from *Cymbopogon* were plotted in accordance with the diameter of the colonies measured after 7 days of incubation. As the concentration increased the diameter of the colony decreased and 3.8mm was measured for the concentration of 5µl/ml. The control sample had 80mm of diameter leading to a MIC at 95.2% (((80-3.8)/80)*100) according to the formula above (Davari and Ezazi, 2017; Li et al., 2016).

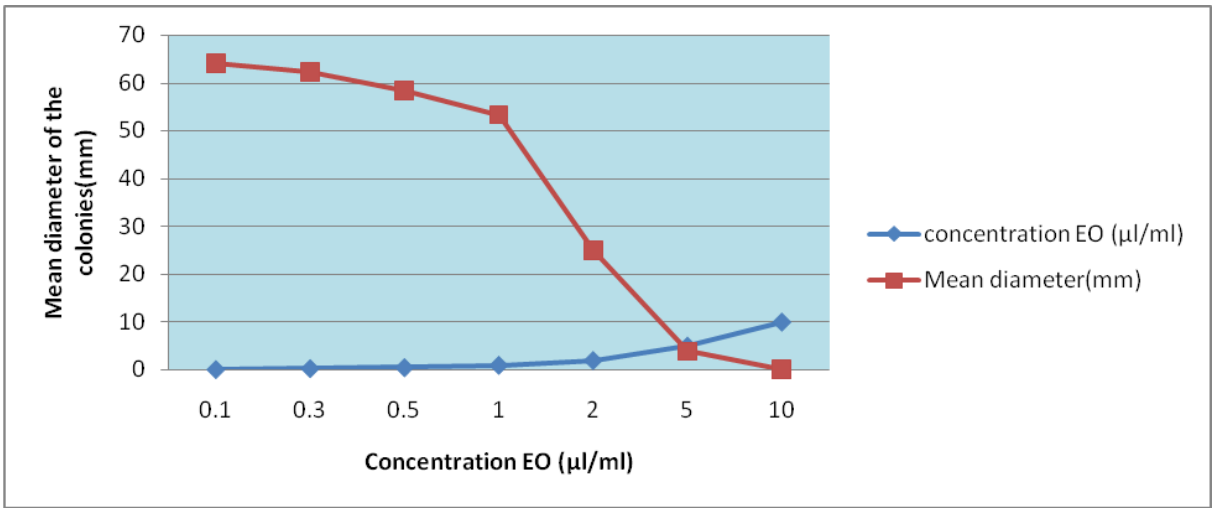

Figure 6. Fungal inhibition per concentration of Cymbopogon nardus

The inhibition was apparent in Figure 7 from the concentration of 30µl/10ml of medium up to the MIC of 50µl/10ml or 5µl/ml, and there was no visible growth at the concentration of 100µl /10ml (10µl/ml). This may be due to the damage of cell wall of the fungal by the essential oil leading to the cytoplasm retraction in the hyphae and the death of mycelium (Sharma and Tripathi, 2008), or mitochondrial dysfunction (Bakkali et al., 2008).

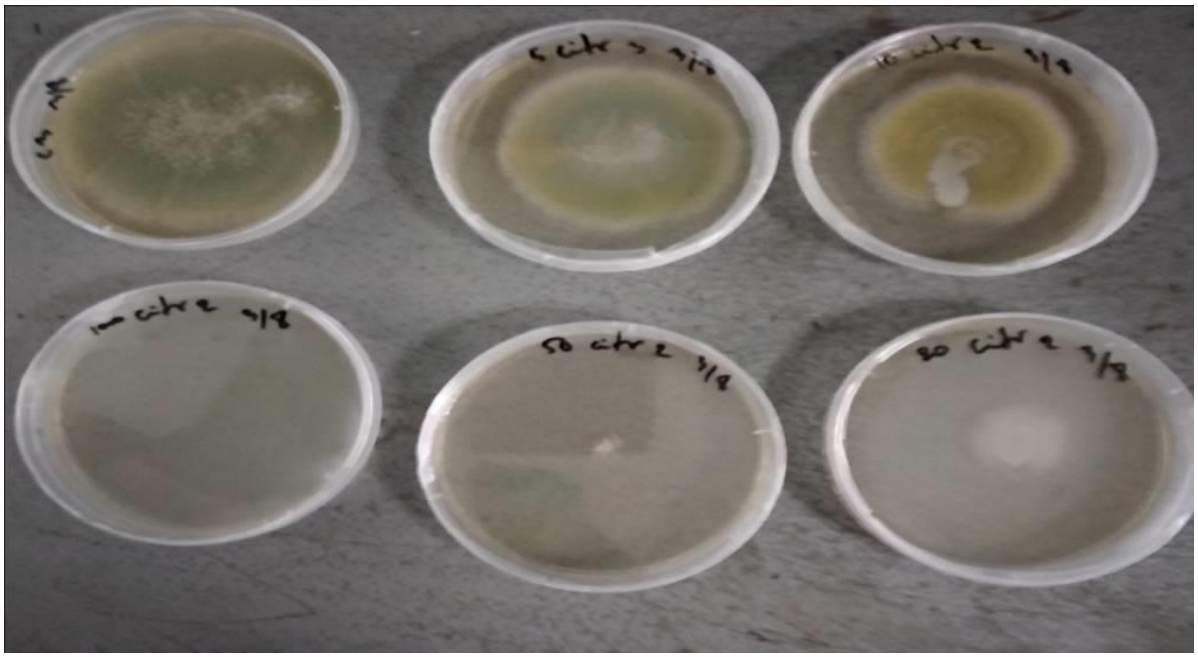

Figure 7.*Cymbopogon nardus* inhibiting fungal vs control (plate 1)

The Table 2 shows the percentages of fungal growth inhibition per concentration and per EOs namely Cinnamon, Basil, Cymbopogon, Mint and Eucalyptus.

Table 2. The minimum inhibitory % of fungal growth per concentration

Essential oils	Scientific name	concentrations (µl/ml) / %inhibition						
		10	5	2	1	0.5	0.3	0.1
Cinnamon	*Cinnamomum verum*	100	100	100	100	100	100	100*
Basil	*Ocimum basilicum*	100	100	100	100	86.9*	74.8	41.3
Citronella	*Cymbopogon nardus*	100	95.2*	68.8	33.3	27.1	22.1	19.8
Mint	*Mentha arvensis*	84.0*	32.3	20.8	14.6	12.9	10.8	10.6
Eucalyptus	*Eucalyptus globulus*	29.4	23.1	18.8	17.3	16.3	12.7	12.1

*% MIC > 80%

Cinnamon, Basil, Cymbopogon and Mint EOs inhibit growth of fungal. Some EOs compounds were more efficient in fungal inhibition. This was consistent with previous studies reporting EOs as natural fumigant and playing a significant role to eliminate storage fungi and increase the shelf life in food (Kohiyama et al., 2015; Prakash et al., 2012; Prakash et al., 2015). A study done by Tian et al. (2012) showed that cinnamon is a powerful inhibitor of spore germination and synthesis of Aflatoxins by *A. flavus* (Tian et al., 2012). The Clinical and Laboratory Standards Institute (CLSI) estimated that an antimicrobial agent as a compound, which can inhibit bacterial and fungal growth at 4µl/ml to be effective (CLSI, 2012). Thus, Cinnamon, Basil and Citronella can be recommended as preservatives. Eucalyptus was less effective, with only 29.4% inhibition at the highest concentration studied of 10µl/ml. This has been reported in previous study done by Davari and Ezazi (2017) where 1-8 cineole, the main compound of eucalyptus essential oil was not effective for fungal growth inhibition (Davari and Ezazi, 2017).

3.3 Sensory Test of Soymilk Flavored with Three Selected Essential Oils

Cymbopogon nardus, *Ocimum basilicum* and mentha were randomly selected for sensory evaluation of soymilk as shown in Table 3. As indicated in the form used for sensory analysis from 6 to 9 score were observed, which meant that the product is liked (Hashmi, 2007). All the soymilk samples had acceptable aroma, color, clarity, taste and overall acceptability except the control for aroma only. The more preferred was mint with score 8 corresponding to 'Like very much' followed by Cymbopogon with a score of 7 (Like moderately) and Basil with a mean score of 6.70. The difference between flavors was significant for aroma, taste and overall acceptability

($P<0.05$).

Table 3. Sensory evaluation of soymilk flavored with 3 EOs at 3 drops per liter

Essential oils	Aroma	Color	Clarity	Taste	Overall acceptability
Cymbopogon	6.20 ±1.61	8.00 ±0.94	7.10 ±1.28	7.10 ±0.99	7.00±0.81
Mint	8.10 ±1.28	7.90 ±0.87	7.70 ±1.25	7.70 ±1.05	8.00 ±0.66
Basil	6.30 ±1.63	7.80 ±1.13	7.30 ±1.49	6.30 ±1.41	6.70 ±1.25
Control	5.80 ±1.03	8.00 ±0.94	7.70 ±1.16	6.10 ±1.52	6.20 ±1.31
Significance:	0.004	0.962	0.665	0.028	0.004

For different concentrations tested using Cymbopogon, Table 4 reports the aroma, color, clarity, taste and overall acceptability of soymilk for 0, 1, 2, 3 drops of EO used to flavor one liter of soymilk.

Table 4. Sensory evaluation of soymilk using 3 concentrations of *Cymbopogon nardus*

Concentrations (drops) Cymbopogon	Aroma	Color	Clarity	Taste	Overall acceptability
0	5.80 ±1.31	7.60 ±1.26	7.80 ±1.03	6.00 ±0.94	6.60 ±0.96
1	6.50 ±0.97	7.70 ±1.25	7.80 ±1.03	6.80 ±0.91	6.90 ±0.73
2	6.60 ±0.84	7.50 ±1.50	7.80 ±1.03	6.80 ±1.03	7.00 ±0.81
3	7.90 ±0.73	7.50 ±1.65	7.60 ±1.17	7.90 ±0.87	7.80 ±0.91
Significance:	0.000	0.987	0.967	0.001	0.025

The three concentrations used to flavor soymilk were accepted with a score above 6 on a scale of 1 to 9 except for the control for aroma, which had a mean score of 5.8. This indicates that flavoring soymilk hides the beany odor and increases the organoleptic attributes of soymilk. Three drops of EOs concentrations were liked very much in soymilk flavoring and the difference between concentrations was significant for aroma, taste and overall acceptability ($P<0.05$). The essential oils showed the capacity as food preservative (Harich et al., 2018; Hyldgaard et al., 2012) and increased acceptability of soymilk.

4. Conclusion

There was significant effect of EOs compounds on fungal growth inhibition and sensory acceptability of soybean milk. Essential oils compounds are good substances for flavoring soymilk to improve organoleptic attributes and also increase the shelf life as they inhibit fungal growth.

Cinnamon and Basil can be promoted for food preservation at lower concentration, while Mint and Citronella should be promoted for food flavoring. The minimum concentration was determined that will serve as baseline for further use in food industry especially in soymilk.

Acknowledgment

This project was funded by Organization for Women in Science for the Developing World (OWSD) and Swedish International Development Cooperation Agency (SIDA) through the PhD fellowship at Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences (CAAS), Wuhan, China. The support from Rwanda Agriculture and Animal Resources Development Board and Jomo Kenyatta University of Agriculture and Technology is highly appreciated. We thank deeply others who contributed for the success of the study.

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Appendix

Sensory evaluation of soymilk

Date:…….....

Put the sign × in the case where your age and gender belong

Age **20 – 30** **31 – 40** **>41**

Gender **Male** **Female**

Instructions: You are provided with different numbered samples of soymilk and you are request to carry out the sensory evaluation. Please rinse your mouth after testing each sample using provided water. Do not seek another person's opinion. Please indicate your degree of liking or disliking on provided parameters by scoring the given samples using the scale below.

Evaluate the samples of soymilk for the attributes given against the following scale:

Like extremely…….9

Like very much…..……8

Like moderately……7

Like slightly……..6

Neither likes nor dislikes.…..... 5

Dislike slightly……..……4

Dislike moderately……..……..3

Dislike very much……...……..2

Dislike extremely……1

Sample	Aroma	Colour	Clarity	Taste	Overall acceptability
222					
223					
221					
224					

Thank you