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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (23): 1751-1757, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.1751.1757

Comparative Study of Antifungal Activities of Six Selected Essential Oils against Fungal Isolates from Cheese Wagashi in Benin

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Abstract: The study has compared the antifungal efficacy of six essential oils, *Cinnamomum zeylanicum*, *Cymbopogon citratus*, *Ocimum gratissimum*, *Pimenta racemosa*, *Syzygium aromaticum* and *Zingiber officinale*, tested in culture medium and in traditional cheese wagashi system against moulds belonging to *Aspergillus*, *Penicillium*, *Fusarium* and *Scopulariopsis* genera in perspective to select the most actives as substitutes of chemical preservatives for wagashi preservation. Results obtained from this work indicated that *Syzygium aromaticum*, *Pimenta racemosa*, *Ocimum gratissimum* and *Cymbopogon citratus* essentials oils were the most actives extracts at *in vitro* assay in decreasing order with strong fungistatic activity against the isolates tested; the pronounced activity was provided by *S. aromaticum* essential oil. The effectiveness of these actives oils on the less sensitive moulds common to these oils showed that, among these extracts that of *Syzygium aromaticum* in particular exerted high sporale reduction against all the strains tested. In sum, *Syzygium aromaticum* essential oil possessed the highest antifungal activity both in culture medium and in wagashi system. Essential oils of *C. citratus*, *O. gratissimum*, *P. racemosa* and above all that of *S. aromaticum*, among the six extracts investigated, were the most promising oils as wagashi additives in substitution of synthetic chemicals ones to extend shelf life time of this by-product of milk for its valorization. Further studies are needed to be performed on the safety of oils for human, the shelf life time of this cheese and its acceptability when treated with essential oils to reduce and control pathogen contamination or native microflora.

Key words: Comparative study, essential oils, antifungal activities, cheese wagashi, Benin

INTRODUCTION

In Benin, a local cheese wagashi obtained by traditional process is a nutritional food containing proteins which allows its use as substitutes of eggs and meat in many dishes of rural than citizens populations. This product could help to fight against nutritional problems due to animals' proteins deficiency (Keke *et al*., 2008; Sessou *et al*., 2012a, b). However, the unsatisfactory conditions of process and preservation of this foodstuff may lead to its contamination by adulterated and pathogenic microorganisms particularly moulds (Aissi *et al*., 2009; ICMSF, 2005). Moulds contamination of wagashi may result in qualitative and quantitative losses of this product. Also, moulds may produced toxic metabolites such as mycotoxins inside wagashi contaminated (Singh *et al*., 2010). Thus, a better control to prevent contamination and spoilage of wagashi during the production, sale and distribution in order to extend its shelf life time and minimize public health hazards is necessary. Synthetic chemicals could be used to control these pathogens. However, these chemical preservatives have been considered as sources of many diseases such as carcinogenic, nephritic and teratogenic threats (Hsouna *et al*., 2011; Barkat and Bouguerra, 2012). The same time, consumers are looking for natural products

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which have less impact on human health and environment and even containing less synthetic preservatives. In the meantime, a lower food salt content is recommended by the World Health Organization, in order to reduce the incidence of cardiovascular disease (WHO, 2002; Angelini *et al*., 2006). For these reasons, alternative methods to control cheese-borne fungi of wagashi and consequently to improve the safety of the product are needed to be performed (Goni *et al*., 2009; Lv *et al*., 2011). Essential oils, secondary metabolites of plants, have recently been recognized as bioactive agents possessing antimicrobial activities and classified as Generally Recognized as Safe substances (ESO GRAS-182.20) by the Food and Drug Administration (Food and Drug Administration, 2005; Hsouna *et al*., 2011; Varona *et al*., 2013). Therefore they could be used to control many pathogenic and spoilage microorganisms in foods (Velazquez-Nunez *et al*., 2013). Several studies have reported *Cinnamomum zeylanicum*, *Cymbopogon citratus*, *Ocimum gratissimum*, *Pimenta racemosa*, *Syzygium aromaticum* and *Zingiber officinale* essential oils to be some of the best agents having broad spectrum potentials to inhibit foodborne pathogens and spoilage organisms (Burt, 2004; Pinto *et al*., 2009; Sessou *et al*., 2012a-c; Yehouenou *et al*., 2012a, b). Their applications as control agents of fungal isolates from cheese wagashi in Benin are widely studies (Sessou *et al*., 2012a-f, 2013). The objective of the present work is to make a comparative study on the antifungal activities of these oils in order to select the most actives as substitutes of chemicals synthetic preservatives for the valorization of this product.

MATERIALS AND METHODS

Essential oils studied: Six plants, *Cinnamomum zeylanicum*, *Cymbopogon citratus*, *Ocimum gratissimum*, *Pimenta racemosa*, *Syzygium aromaticum* and *Zingiber officinale* collected in Benin and identified by Doctor YEDOMOHAN of National Herbarium of Benin were hydrodistilled and the essential oils obtained were analyzed by GC/MS and GC/FID. The major compounds in these oils are reported in Table 1.

Strains of filamentous fungi tested: The strains investigated were constituted of adulterated and pathogenic moulds *A. flavus*, *A. niger*, *A. tamarii*, *A. terreus*, *Fusarium poae*, *F. verticillioides*, *P. citrinum*, *P. griseofulvum*, *Aspergillus aculeatus*, *A. ustus*, *Penicillium brevicompactum* and *Scopulariopsis brevicaulis* identified by morphological and microscopic characteristics (Sessou *et al*., 2012a).

Table 1: Major components of essential oils studies

Essential oils	Botanical family	Major components	%	References
Pimenta racemosa	Myrtaceae	Eugenol	51.1	Sessou *et al*. (2012b)
		Myrcene	25.1	
Ocimum gratissimum	Lamiaceae	Thymol	28.1	Sessou *et al*., 2012a, c)
		Para-cymene	21.3	
		γ-terpinene	16.5	
Cinnamomum zeylanicum	Lauraceae	(E)-cinnamyle acetate	39.9	Sessou *et al*. (2012c)
		(E)-cinnamaldehyde	25.0	
		Benzyle benzoate	20.5	
Zingiber officinale	Zingiberaceae	Zingiberene	40.7	Sessou *et al*. (2012d)
		geranial	8.9	
Syzygium aromaticum	Myrtaceae	Eugenol	75.2	Sessou *et al*. (2012e, 2013)
		Trans-β-caryophyllène	12.0	
Cymbopogon citratus	Poaceae	Geranial	44.5	Sessou *et al*. (2012f)
		Neral	31.5	

Preparation of conidial suspension: Conidial suspension concentration of mould was determined by a haemocytometer and the suspension was diluted with 0.05% Tween 80 solution to give a final concentration of 10^8 spores mL^{-1} approximatively (Gandomi *et al*., 2009).

Antifungal assay in culture medium: The test was performed by the agar medium assay described by Tatsadjieu *et al*. (2009) with different concentrations of essential oil, 200, 400, 600, 800 or 1000 mg L^{-1}. The MGI (Mycelia Growth Inhibition) was determined on each species for each oil. The Minimal Fungicidal Concentration (MFC) values were determined by the method described by Angelini *et al*. (2006).

Antifungal assay in cheese wagashi foodsystem: The procedure performed is that of Sessou *et al*. (2012f). About 10 g of sterile cheese wagashi was added to 90 mL of 0.1% peptone in stomacher bags and homogenized for 2 min in a stomacher. Essential oil was added to the cheese mixture to achieve final concentrations wished. The inoculum was mixed thoroughly with the cheese mixture by gently squeezing the bags by hand and the concentration of mould in the cheese determined at 0 h and 1, 2, 3, 4, 7, 10 and 14 days using the serial dilution and spread plate technique. The assay was conducted on moulds which were less sensitive at *in vitro* assay and common to actives essential oils in order to compare their activities.

Statistical analysis: Statistical analysis was carried out with SAS software (SAS, 1989) and several procedures were used. The procedure of generalized linear models (Proc-GLM) was used for the analysis of the variance and the means of inhibition percentage of three independent replicate trials were then calculated and compared by a Z-test using Statistica version 6.0 (StatSoft, 2010).

RESULTS

Six essential oils obtained from *Cinnamomum zeylanicum*, *Cymbopogon citratus*, *Ocimum gratissimum*, *Pimenta racemosa*, *Syzygium aromaticum* and *Zingiber officinale* were compared based on their ability to control foodborne pathogens and spoilage moulds. Among the essential oils tested, those of *Syzygium aromaticum*, *Pimenta racemosa*, *Ocimum gratissimum*, *Cymbopogon citratus* exhibited broad-spectrum antifungal activities in culture medium against the species of moulds investigated; while those of *Zingiber officinale* and *Cinnamomum zeylanicum* showed less antifungal activity, they inhibited the mycelial growth in a few fungal strains (Table 2 and 3). *Syzygium aromaticum* essential oil was the most effective as antifungal agents, followed by *Pimenta racemosa*, *Cymbopogon citratus* and *Ocimum gratissimum* essential oils in decreasing order at *in vitro* test (Table 3). In all cases the inhibitory effects of these oils were linked to dose tested ($p<0.05$). Based on minimal inhibitory concentrations of these extracts, those of *Syzygium aromaticum* and *Pimenta racemosa* were the most actives to fight against *Aspergillus* and *Penicillium* species. To fight against *Fusarium* species, *S. aromaticum* oil is the most recommended followed accessory by *Pimenta* oil whereas *Ocimum gratissimum*, *Cinnamomum zeylanicum*, *Cymbopogon citratus* and *Syzygium aromaticum* constituted the most actives control agents of *Scopulariopsis brevicaulis* specie. *Aspergillus tamarii* was the less sensitive common specie to essential oils of *Syzygium aromaticum*, *Ocimum gratissimum* and *Cymbopogon citratus* while *Penicillium citrinum* was the less sensible common specie to *Syzygium aromaticum*, *Pimenta racemosa* and *Cymbopogon citratus* essential oils at *in vitro* test. *Penicillium brevicompactum* and *Fusarium verticillioides* were the less sensitive common species to *Pimenta racemosa* and *Ocimum gratissimum* essential oils in culture medium test.

Table 2: Mycelial growth inhibition, fungistatic and fungicidal activities of six essential oils investigated

Essential oil	Mycelial Growth Inhibition (%)												
(mg L^{-1})	*A. acu*	*A. fla*	*A. nig*	*A. tam*	*A. ter*	*A. ust*	*F. poa*	*F. vert*	*P. brev*	*P. cit*	*P. gri*	*S. brev*	Significance
Syzygium aromaticum													
400	69.6±2.8^{d}	100a (FS)	100a (FS)	82.1±3.2^{bc}	100a (FS)	100a (FS)	85.8±2.2^{b}	100a (FS)	70.1±0.5^{d}	81.1±0.4^{c}	100a (FS)	100a (FC)	+++
600	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FC)	ns
800	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FC)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FC)	ns
1000	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FC)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FS)	100a (FC)	ns
Cinnamomum zeylanicum													
600	35.7±0.4^{e}	14.9±0.4^{g}	7.1±0.3^{h}	16.7±1.6^{fg}	100a (FC)	17.8±0.7^{f}	92.6±1.3^{b}	89.4±0.3c	92.3±0.8^{b}	70.3±0.3^{d}	88.9±0.3^{c}	100a (FS)	+++
800	50.0±0.2^{f}	17.9±0 .6^{i}	45.2±0.8^{g}	22.6±0.2^{h}	100a (FC)	53.6±0.1^{e}	96.7±0.24^{b}	92.5±0.2c	96.1±0.4^{b}	75.7±1.3^{d}	100 Fs^{a}	100a (FC)	+++
1000	59.5±0.8^{c}	35.7±1.2^{d}	79.8±1.9^{b}	30.4±0.4e	100a (FC)	79.8±1.8^{b}	100a (Fs)	100a (Fs)	100a (FS)	79.7±0.2^{b}	100a (Fs)	100a (FC)	+++
Zingiber officinale													
1000	41.7±0.3^{e}	23.8±0.9^{i}	5.9±0.5^{k}	11.9±0.5^{j}	87.1±2.6^{c}	45.2±3.8^{g}	39.3±0.7^{h}	66.1±1.4^{f}	77.4±2.1^{e}	79.7±0.1^{d}	100a (FS)	97.6±0.1^{b}	+++
Pimenta racemosa													
600	100a (Fs)	66.4±0.2d	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	80.3±0.1^{c}	64.1±0.2e	96.6±0.7b	80.4±1.1c	100a (Fs)	100a (Fs)	+++
800	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	ns
1000	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	100a (Fs)	ns
Cymbopogon citratus													
800	100a (FS)	69.2±0.7^{c}	100a FS	57.1±0.7^{d}	100a (FS)	39.9±2.3^{f}	100a (FS, FC)	38.6±1.1^{f}	100a (FS)	89.2±0.3^{b}	50,9±2.1^{e}	100a (FC)	+++
1000	100a (FS)	100a (FS)	100a (FC)	100a (FS)	100a (FC)	95.2±0.5^{b}	100a (FC)	100a (FC)	100a (FS)	100a (FS)	68.9±0.5^{c}	100a (FC)	+
Ocimum gratissimum													
800	100a (FS)	95.8±1.5^{c,d,e}	95.2±0.9^{d}	96.4±0.2^{c}	100a (FS)	98.8±0.6^{b}	100 a (Fs)	91.1±0.9^{f}	94.9±0.2^{e}	100a (Fs)	100 a (Fs)	100a (FC)	++
1000	100a (FS)	100 a (Fs)	100a (FS)	100a (Fs)	100a (Fs) (FC)	100a (FS)	100 a (Fs)	100 a (Fs)	100a (FS)	100a (Fs)	100 a (Fs)	100a (FC)	ns

FS: Fungistatic activity, FC: Fungicidal activity, data in the line followed by different letters are significantly different ($p<0.001$), The values are means of the repetitions±standard deciation, Key: *A. Acu*: *Aspergillus aculeats*, *A. Fla*: *Aspergillus flavus*, *A. Nig*: *Asperillus niger*, *A. Tam*: *Aspergillus tamarii*, *A. Tecitrinum*, *P. Gri*: *Penicillium griseofulvum*, *S. Brev*: *Scopulariopisi brevicaulis*, +++: Significative ($p<0.001$), ns: Non significative. The results presented in this table are those significant for comparative activites of oil studied

Table 3: Percentage of studied fungal strains inhibited at *in vitro* test

Essential oils (mg L^{-1})	Percentage of fungal strains inhibited (%)					
	S. aromaticum	*C. zeylanicum*	*Z. officinale*	*P. racemosa*	*C. citratus*	*O. gratissimum*
1000	100.00	50.00	8.33	100.00	83.30	100.00
800	100.00	25.00	0.00	100.00	50.00	50.00
600	100.00	16.67	0.00	58.31	16.67	8.33
400	58.31	8.33	0.00	33.32	8.33	8.33
200	8.33	0.00	0.00	0.00	0.00	0.00
Grade	1.00	5.00	6.00	2.00	4.00	3.00

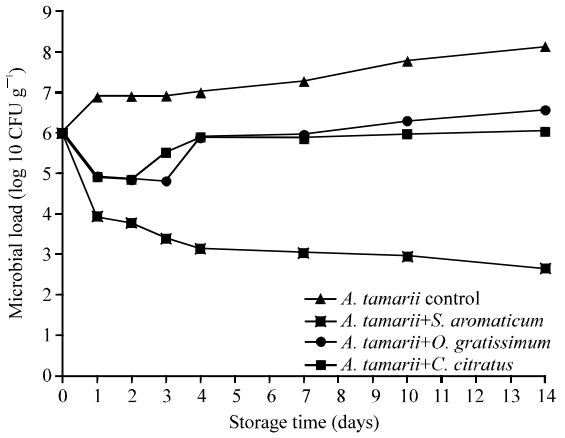

Fig. 1: Inhibition of *Aspergillus tamarii* in traditional cheese wagashi by *S. aromaticum, C. citratus* and *O. gratissimum* essential oils at concentration of 1000 mg L^{-1}

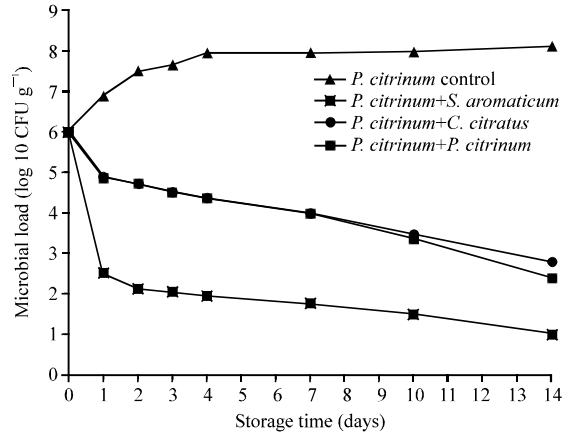

Fig. 2: Inhibition of *Penicillium citrinum* in traditional cheese wagashi by *S. aromaticum, C. citratus* and *P. racemosa* essential oils at concentration of 1000 mg L^{-1}

The active oils (*S. aromaticum, Pimenta racemosa, Ocimum* and *Cymbopogon citratus*) at *in vitro* assay were tested against their common less sensitive moulds in cheese wagashi and showed high sporale reduction of *Aspergillus tamarii* and *Penicillium citrinum* (Fig. 1 and 2) with *Syzygium aromaticum* oil. In fact, quantum of *Aspergillus tamarii* was reduced to 1.02 log 10 CFU g^{-1} of its load at fourteenth day of storage at 1000 mg L^{-1} with *Syzygium aromaticum* essential oil whereas the spore load of the same strain was less reduced, respectively to 2.79 log 10 CFU g^{-1} and 2.4 log 10 CFU g^{-1} by essential oils of

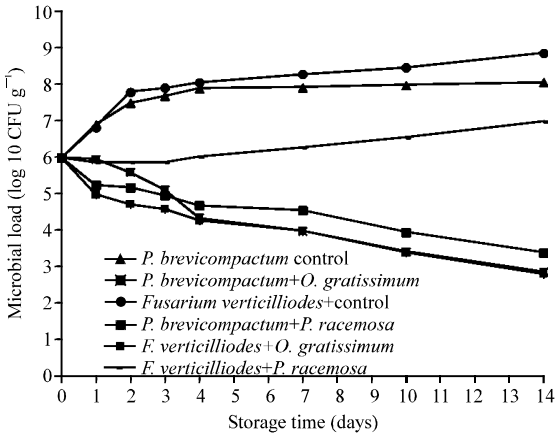

Fig. 3: Inhibition of *P. brevicompactum* and *F. verticillioides* in traditional cheese wagashi by *P. racemosa* and *O. gratissimum* essential oils at concentration of 1000 mg L^{-1}

Cymbopogon citratus and *Pimenta racemosa* at the same concentration the day 14. At the same time, the spore load of the control of *Aspergillus tamarii* without essential oil has increased of 6 log 10 CFU g^{-1} to 8 log 10 CFU g^{-1} on the fourteenth day of storage. The same remarks were observed with these oils on *Penicillium citrinum* specie. Figure 3 showed that essential of *Ocimum gratissimum* were more active on *Fusarium verticillioides* than that of *Pimenta racemosa* in cheese wagashi foodsystem. The same remark was observed for these oils on *Penicillium brevicompactum*. In fact, microbial load of *Fusarium verticillioides* was reduced to 2.83 log 10 CFU g^{-1} of its load at fourteenth day of storage at 1000 mg L^{-1} with *Ocimum gratissimum* whereas in presence of *Pimenta racemosa* essential oil at the same concentration, the quantum of *Fusarium verticillioides* increased until to 6.99 log 10 CFU g^{-1} the day 14. The same observations were noted for these oils against *Penicillium brevicompactum* where the reduction of spore load of this mould is pronounced with *Ocimum gratissimum* oil than that of *Pimenta* extract. In sum, these experimentations in wagashi food system revealed that the activity of *S. aromaticum* is higher than that of *P. racemosa* essential oil and this last than that of *Cymbopogon citratus* on *Penicillium citrinum*. On *Aspergillus tamarii*, *Syzygium aromaticum* activity is higher than that of *Cymbopogon citratus* essential oil and this last than that of *Ocimum gratissimum*. The activity of *O. gratissimum* was significative on *Fusarium verticillioides* than that of *P. racemosa* essential (Table 4).

Table 4: Residual spores load of moulds in cheese wagashi treated with 1000 mg L^{-1} of essential oils at the end of storage time (14th day)

		Penicillium citrinum		
Essential oils	N	Mean of residual spore load (log 10 CFU g^{-1})	Standard deviation	Significance
C. citratus	3	2.79[a]	0.09	+++
P. racemosa	3	2.40[b]	0.09	
S. aromaticum	3	1.02[c]	0.08	
Aspergillus tamarii				
C. citratus	3	6.03[b]	0.02	+++
O. gratissimum	3	6.56[a]	0.08	
S. aromaticum	3	2.66[c]	0.15	
Penicillium brevicompactum				
O. gratissimum	3	2.89[a]	0.05	ns
P. racemosa	3	3.41[a]	1.08	
Fusarium verticillioides				
O. gratissimum	3	2.83[b]	0.21	+++
P. racemosa	3	6.99[a]	0.01	

DISCUSSION

Food security is a major preoccupation both for consumers and the food industry, particularly as the number of food-associated infections cases continues to rise (Alzoreky and Nakahara, 2003). According to World Health Organization, about 30% of people in industrialized countries, annually, suffer from foodborne diseases (WHO, 2002). Microorganisms play a major parts in stored food contamination by deterioring them quantitatively and qualitatively. These strains are mainly controlled by synthetic fungicides which can often be problematic when used for treatment because of their high residual toxicity to mammals (Bakkali *et al*., 2008). The use of synthetic chemicals to fight against microbial strains has been discouraged due to their negative effects on food and human health. In addition to these impacts on food and consumers' health, microorganisms can also develop resistance to synthetic controlling agents. The use of higher concentrations of synthetic agents to control the microbial resistance increase the risk of high content of toxic residues by the products (Barkat and Bouguerra, 2012). Furthermore, the limitations of some synthetic food additives use by food industry and regulatory agencies have led to great importance to natural antimicrobial components, especially essentials oils which are secondary metabolites of plants (Singh *et al*., 2010). Essential oils as well as their constituents possess a broad range of activities among which antibacterial and antifungal activities are largely investigated (Ngueфack *et al*., 2007). Food mycoflora has been traditionally few studies contrary to bacterial flora of foodstuffs. Better, essential oils have received great attention concerning their antibacterial activities whereas the effects of these substances on food spoilage and pathogenic moulds were few investigated (Burt, 2004; Angelini *et al*., 2006). According to our studies essential oils of *Syzygium aromaticum*, *Pimenta racemosa*, *Ocimum gratissimum* and *Cymbopogon citratus* were the most harmful extracts against species of moulds investigated. The activities of these oils could be linked to their richness in phenolic and aldehydic compounds. In fact, considering *Zingiber officinale* and *Cymbopogon citratus* essential oils, both contained geranial, in minor percent in *Z. officinale* essential oil and in large proportion in *Cymbopogon citratus* essential oil which was the more active of these two oils. The antifungal activity of each one of these oils may be linked to their content in geranial which may possess a high antifungal activity. Considering essential oils of *Pimenta racemosa* and *Syzygium aromaticum* belonging to Myrtaceae family, both contained eugenol in large proportion with that of *Syzygium aromaticum* in high percentage than that of *Pimenta racemosa*. From these two oils *S. aromaticum* extract was the more active. The activity of these two oils may be due to their content in eugenol which is a phenolic compound generally possessing antimicrobial activity (Burt, 2004, Lopez-Reyes *et al*., 2010). The two oils containing eugenol possessed high antifungal activity than those containing geranial. The antifungal activity of eugenol may be higher than that of geranial. Also, the composition of *Ocimum gratissimum* in thymol, a phenolic compound may be at the origin of the antifungal activity of this oil. The antifungal activity of this last extract than that of *Pimenta* on *Fusarium verticillioides* may be link to the fact that thymol has more negative effect on cellular membrane of *Fusarium* species than that of eugenol. These hypotheses relative to the antifungal activity of thymol, geranial and eugenol must be confirmed by further studies in which antifungal activity of each molecular will be only tested on each genera of strain *Aspergillus*, *Penicillium* and *Fusarium*. Altogether, essential oils of *Syzygium aromaticum*, *Pimenta racemosa*, *Ocimum gratissimum* and *Cymbopogon citratus* were the most promising antifungal agents which could be used for traditional cheese wagashi preservation in Benin.

CONCLUSION

The present investigation concluded that *Syzygium aromaticum*, *Pimenta racemosa*, *Ocimum gratissimum* and *Cymbopogon citratus* possessed differentially high fungistatic activity against *Aspergillus*, *Penicillium*, *Fusarium* and *Scopulariopsis brevicaulis* genera and could be used as wagashi preservatives in replacement of synthetic chemicals ones for the valorization of this foodstuff. The use of these natural substances as antifungal agents, may be of

interest given that essential oils are of natural origin and safer for human health and the environment and there is less chance that the pathogenic microorganisms will develop resistance. Toxicity of these extracts and acceptability of wagashi treated with these oils are need to be investigated.

ACKNOWLEDGMENTS

The authors are grateful to Doctor SEBASTIEN TINDO DJENONTIN in memoriam (He was coldly shot by thugs at his home March 11th, 2013; May he rest in peace) for its scientific contribution. They are also thankful to University Council of Development (CUD) for its financial support.

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