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Antimicrobial Activity of Methanolic Extract from Clove and the Test of Preservative Power of Ras Cheese

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ABSTRACT

The effect of methanolic extract from Clove (*Syzygium aromaticum* L.) and sodium sorbate (SS) on growth of certain fungal, bacteria strains and Ras cheese sampls during storage were examined. Ras cheese made from standardized cow's milk (4% fat) with a modified process. During production curd cheese, four groups were formed: first group taken after salting for 12 days, without Clove extract (CE), as control, second group (T1) add CE to curd cheese, third group (T2) add SS to curd cheese, and fourth group (T3) add CE on surfaces of each cheese wheel. After completely salting process, the cheese ripened at 15±2ºC and about 85% relative humidity for 3 months. All treatments evaluated microbiologically at fresh, and after one, two & three months. The samples we took from the exterior (Ex) and inside (In) of cheese wheel. The control samples had higher counts of yeast & fungal and TBC in S and M of cheese than that of other treatments. The counts of yeast & fungal and TBC of S and M of control samples increase with increasing of storage, while cheese treated with CE & SS decrease with increasing storage periods up to end of storage. Moreover, the coliform bacteria were not detected up to the end ripening. Generally, the results showed that the SS had higher antimicrobial effect than that with CE on growth of yeast & fungal and general microorganism especially the responsible for ripening cheese during storage.

Keywords: Ras cheese, Microbiological examination, Methanolic extract Clove, Fungal, Yeast and bacteria strains.

INTRODUCTION

Romy cheese is call an Egyptian Ras or hard cheese manufactured by cows and buffalo's milk, or a combination the two. It is a popular dairy product in Egypt and the Arab world (El-Fadaly *et al.*, 2015). The depravation of Ras cheeses inside dairy produced labs in Egypt is familiar during the ripening process, where a layer of fungi and yeasts grow on the surfaces of the cheese wheels, due to the lack of care in the manufacturing and production. The processes and the failure to achieve the regular conditions of the ripening process leading to high economic losses, as well as, the production of some of these types of fungi toxic compounds that are not safe for health, called aflatoxins, which cause many diseases. When consume contaminated products, negative impact on the consumers health as a result of their exposure to mycotoxins, especially that produced by *Aspergillus* spp. (Mohsen, 2012). Therefore, the microbial growth decontamination and prevention on Ras cheese wheels surface is of great importance. Many methods used natural preservatives to reduce and inhibition of Mould and yeast growths on Egypt Ras cheese, several previous reports were focused on the antimicrobial properties of different plant essential oils specially Clove oil or methanolic extract from it in the production of hard and soft cheese (El-Fadaly, *et al.*, 2015).

Syzygium aromaticum, and well-known for its as Clove, belongs to family Myrtaceae, which is native to the Indonesian Maluku Islands but has recently been farmed elsewhere in the world (Batiha et al., 2019). The commercial component of the clove tree, which consists of leaves and buds, starts to produce flowering buds four years after planting. Following that, they are harvested either manually or with the aid of a natural phytohormone during the pre-flowering stage (Cortés-Rojas *et al.*, 2014). Traditional spice clove has been used to preserve food and has a number of pharmacological effects, including: It is abundant in sesquiterpenes, monoterpenes, hydrocarbons, and phenolic compounds, among other phytochemicals. The three most important phytochemicals found in clove oil are eugenyl acetate, eugenol, and -caryophyllene. Pharmacologically, it has been tested against a variety of pathogenic bacteria, parasites, and microbes, including hepatitis C and Plasmodium, Babesia, and Theileria. Numerous studies have shown that eugenol has antibacterial, antiviral, antifungal, anticancer, antiseptic, antidepressant, spasmodic, and anti-inflammatory properties that make it effective against a variety of pathogenic bacteria, including methicillin-resistant S. aureus and S. epidermidis (Batiha *et al.*, 2020). In this study, we examined the antimicrobial activities of methanolic Clove extract, and study its effect on microbial decontamination and preservation of Ras cheese during storage periods at 15±2ºC and about 85% relative humidity for three months.

MATERIALS AND METHODS

Fresh whole cow's milk acquired from the Animal Herd Production Division, Al-Azhar University's Faculty of

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Agriculture (Assiut Branch). Whole fresh milk that has been skimmed cow's using a laboratory centrifuge set at 3000 x g for 10 min at 20 °C, milk is made from defatted cream. Enzyme sources (Microbial rennet, 1 N): used as a powder that was purchased from DSM (France) and had a brand name (Fromase R 2200). 3g were dissolved to make of powder rennet in 100 ml distilled water; and used at a rate of 1.0 ml/L milk (0.03 g/L milk) Purchasing salt (commercial sodium chloride) from the El-Nasr Company for salt (Alexandria, Egypt). Calcium chloride obtained from Caso Co., Italy, imported by El-Zawaowi Co. El-Gesh Street, Cairo, Egypt. Fresh whole Clove samples from Edfu, Aswan, Egypt. Sodium sorbate from local market.

Preparation of methanolic extract:

Clove methanolic extract were prepared according to Wilson (1995) as follow:

Fresh whole clove was ground in a laboratory grinder (MJ-176P) and kept in opaque screw-tight jars until use. 25 g of chopped clove were combined with 150 ml of methanol, and the mixture was then stirred in a shaker incubator for 8 hours. It was then funnelled through Whatman paper and into a conical flask. The filtrate in the conical flask was extracted by using a rotavapor machine. In order to prevent the bioactive chemicals from being damaged by high temperatures, the filtrate was dried at a temperature below 50°C. The dark colored oily extract obtained is known as crude methanolic extract. Until the analysis was finished, the sample extract was stored at 4°C, the temperature of the refrigerator.

Ras cheese manufacture:

Ras cheese was prepared in accordance with Abdel Tawab (1963) with the following modifications:

Cow's milk (4% fat) heated to 75ºC for 20 s, and then cooled to 40ºC. Lactic acid starter {*Str. thermophlus, Lac. delbrueckii spp. bulgaricus* and *Lac. casei spp. casei* (1:1:1)} were used 1% and added at 30ºC, Add rennet 0.03 g/l milk after completely blending, and then wait 30 minutes for the acid to develop. After coagulation within 45 min, The whey was then drained to the level of the curd, which had an acidity of 0.14%, after the curd had been sliced into small cubes and the temperature elevated to 45oC in 15 minutes. After cooling the curd, 2 kilogramme of salt was added for every 100 kg of milk. For the first two hours, the mould and press were loaded with 160 Ibs. Overnight, the weight was increased to 1000 Ibs for pressing. Dry salt was then applied to both sides of the cheese wheel to finish drying it. The procedure of salting certain sites took 12 days to complete. Keep portion without any additive as control samples, and then adding both CE 100mg/1kg and SS 1.5g/1kg curd cheese for the two treatments T_1 and T_2; respectively following a thorough salting procedure, cheese wheels received T3 treatment, which painted clove extract on the surface. All treatments make ripe at 15±2ºC and about 85% relative humidity, and stored for 3 months, cheese wheels not waxed. Samples were taken initially at fresh and after1st, 2nd and 3rd months for analysis.

Antimicrobial activity of Clove methanolic extract:

Determination of antifungal activities of Clove methanolic extract:

Potato dextrose agar (PDA) media (200 g of potato, 20 g of dextrose and 20g of agar) was thawed at 45ºC and poured onto 9 cm Petri dishes. After cooling at room temperature, the dishes were fertilized with uniform discs of the fungus. Two tested fungi genus; AF and AN against the tested Methanolic extract 1 mg/10 ml di-methylsulphoxid (DMSO). The experiment was made in triplicate for each fraction. The dishes were incubated for 7 days at 25±3ºC, then, the inhibition zone was measured as mentioned by McKee *et al.* (1990).

Determination of antibacterial activities of Clove methanolic extract:

The sample was extracted from Clove with methanol and then the solvent was removed. Methanolic extract 1 mg/10 ml di-methylsulphoxid (DMSO) was added to the sterilized, cooled PDA-medium. In addition, the inoculation with *E. coli* and salmonella were done and all dishes were incubated at 37ºC. After 2 days the dishes were examined and the growth was determined with compared to control (Fardiaz, 1989).

Qualitative examination of secondary metabolites of Clove methanolic extract:

Secondary metabolites such tannins and saponins in the Clove methanolic extracts were determined as described by Madankumar and Pari (2020).

Quantitative determination of secondary metabolites of Clove methanolic extract:

Secondary metabolites such as tannins and saponins in the Clove methanolic extract were determined as described in literatures (Zheng and He, 2014; Makkar et al., 2008).

Extraction and determination of total phenolic compounds (TPCs):

The total phenolic compounds were extracted from sample (0.5 g) by refluxing with 30 ml of methanol containing 1.0% HCl for 10 min and centrifugation at 5000 rpm for 10 min. Using a standard method developed by Singleton and Rossi (1965), the content of all phenolic components in the methanolic extracts was quantified as gallic acid equivalents.

Microbiological examination of Ras cheese:

Using the "poured dishes" technique, all cheese samples were serially diluted in one ml and inoculated into a dish containing potato dextrose agar (PDA) media (three replicates). Each plate received approximately 50 cc of PDA media at about 50°C, which was then properly mixed and allowed to solidify. The dishes were incubated for 5 days at 25°C. Each dish's formed colonies were counted after the incubation time. According to APHA (1998), the total bacterial count of Ras cheese was calculated using nutritional agar media (Difco, 2009), and the mean count of plates was recorded. It comprises of (g/L):

Beef extract 3 g; peptone 5 g; agar 15 g; distilled water 1 L and sterilized by autoclaving at 121ºC for 15 min. The pH was adjusted to 6.8, dishes were incubated at 37°C for 48 h, before counting.

Coliform of cheese samples were determined according to American public Health Association (1992), using VRBA media, its consists of (g/L):

crystal violet (0.002 g), neutral red (0.03 g), glucose (10 g), peptone (7 g), sodium chloride (5 g), bile salts "No. 3" (1.5 g), agar (12 g), and purified water (1000 ml). The final pH was adjusted to 7.0 ± 0.2. All ingredients were allowed to soak for 15 min, and then boiled with shaking until completely dissolved. The suspension was autoclaved

at 121°C for 15 minutes to sterilise it. The dishes were incubated at 37°C for 48 hours after being poured.

RESULTS AND DISCUSSION

Yield of methanolic extracted from Clove:

According to Table 1, data received indicated that a high methanolic extract yield (25%) was recorded from the Clove. Our findings are fairly consistent with Rungnapa's (2019) and his colleagues who found that the aqueous and methanolic extract of dried Clove bud (20 g) were 23 and 34%; respectively.

Table 1. Yield of methanolic extracted from Clove.

Yield of methanolic extract (%)	25%

Qualitative examination of the secondary metabolites of Clove methanolic extract:

The Clove methanolic extract was examined for qualitative detection of secondary metabolites, and Table 2 displays the outcomes. It could be noticing that, the Clove contains three different groups of secondary metabolites including phenolic compounds, tannins and saponin.

Table 2. Screening of secondary metabolites in the Clove methanolic extract.

Sample	Total phenolic	Tannins	Total saponin
Clove	+	+	+

Quantitative determination of the secondary metabolites in the Clove methanolic extract:

Total Phenolic compounds, tannins and saponins in Clove methanolic extract were determined and the results are given in Fig. (1). each gram of the Clove methanolic extract was found to contain appreciable amounts of TPCs (240.25±0.75 mg) and tannins (56.5±0.56 mg) in addition to saponins (40.25±1.67 mg). These substances, which are abundantly found in the plant world and have been reported to be effective against a variety of species, aid in the defence against predators and pathogens (Gawri and Upadhyay, 2012).

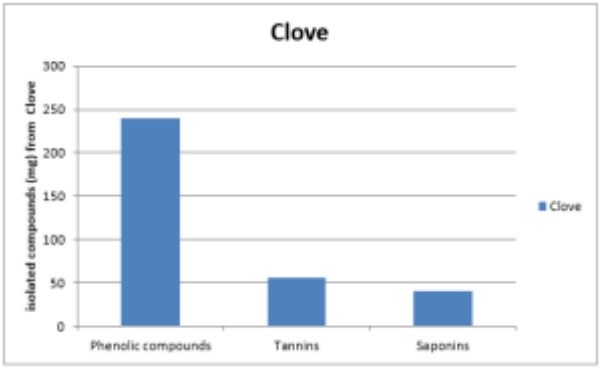

Fig. 1. Levels of (TPCs), tannins and saponins in the Clove methanolic extract mean ± SEM (n= 4)

Effect of Clove methanolic extract on growth of *Aspergillus flavus* (AF) and *Aspergillus Niger* (AN):

The impact of methanolic clove extract on fungal growth namely AF and AN are given in Fig 2. Our results showed existence of inhibition zones with different Clove methanolic extents and the higher concentrations led to wider zones. In addition, the data revealed that the growth of AF is less resistant than AN with another words the cells of AF is more sensitive. Similar studies were carried out by Rungnapa *et al.* (2019) sought to examine the antifungal effects of betel leaf and clove bud aqueous and methanolic extracts against AF and to determine the viability of using the plant extract to prevent the growth of fungi while storing dried chili. The capability of antifungal aqueous and methanolic extracts of Clove bud and betel leaf showed a dose dependent manner, which methanolic extracts of both plants exerted 2-5 times higher antifungal activity against AF TISTR 3151 in PDA as compared with the aqueous extracts. Moreover, Yassin *et al* (2020) found that Clove ethyl acetate extract exhibited the highest antifungal activity against *C. tropicalis*, *C. albicans* and *C. glabrata*.

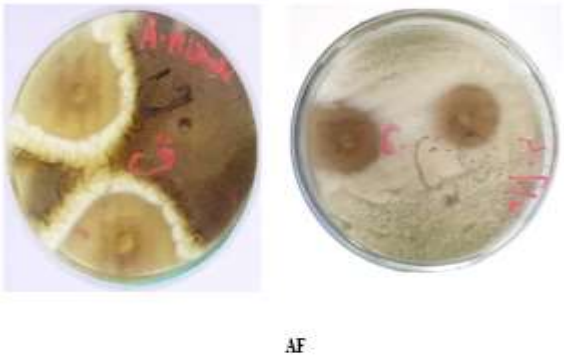

Fig. 2. Effect of Clove methanolic extract on fungal growth of AF and AN.

When used against *C. tropicalis*, *C. albicans,* and *C. guilliermondii,* clove oil was said to have the strongest antifungal effects (Kumar *et al.*, 2012). Strong antifungal activity was also demonstrated by encapsulated clove oil against Fusarium oxysporum (Estrada-Cano *et al.*, 2017) at a dosage over 0.070%, and antimicrobial effects on meat products (Kaur and Kaushal, 2019).

Effect of Clove methanolic extract on *E. coli* and *Salmonella* growth:

Results of effect Clove methanolic extracted on growth of *E. coli* and *Salmonella* are given in Fig. 3 showed that the methanolic extract of cloves revealed positive results on inhibition *E. coli* growth compared to *Salmonella* for antibacterial activity. Additionally, it is clear from the findings that when extract concentration grew, the diameter of inhibition also did so. The findings of the current study concur with those of Saeed *et al* (2013), they found that clove methanolic extract has good antibacterial action against E. coli utilising aqueous and methanol extracts. The strong antibacterial activity of clove extracts in ethanol and methanol against Pseudomonas aeruginosa and E. coli has been documented (Garba *et al.*, 2019).

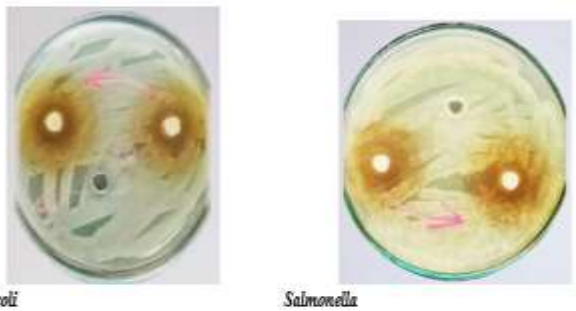

Fig. 3. Effect of Clove methanolic extract on bacterial growth of on *E. coli* and *Salmonell*a.

Clove methanolic extract was demonstrated to have a higher inhibitory effect than pure eugenol at the same concentration on five bacteria responsible for urinary tract infections. This inhibition was broad spectrum (Gram-positive and Gram-negative) (Rosarior et al., 2021). Methanol extract has been shown to have antibacterial efficacy against Klebsiella pneumonia (Hemalatha *et al*., 2016).

The microbiological properties of Ras cheese samples during storage:

The information shown in Table 3 evidenced this, samples of Ras cheese treated with (Clove methanolic extract & sodium sorbate) and untreated (control) for storage periods up to three months had different counts of TBC and yeast & fungal.

The findings demonstrated that, counts of TBC and yeast & fungal were quite high in control samples in all treatments, the counts of yeast and fungal of fresh samples were lower in T1 and T2 than that of control samples and T3, with counts of 2.74 & 3.10 and 4.05 & 4.05 Log cfu; respectively in samples taken from the middle. The TBC of cheese taken from exterior and inside of fresh cheese for C, T_1, T_2 and T_3 were (4.01 & 5.91), (4.01 & 5.84), (3.74 & 4.11) and (4.00 & 5.21) Log cfu/g; respectively. The counts of surface lower than that of middle in all treatments. The surface of control samples had not any counts of yeast & fungal in all treatments.

Table 3. Microbiological analysis of Ras cheese wheel samples from both treated and untreated cheese during storage times of up to three months.

Parameters	Storage periods (Month)	C		T1		T2		T3	
		Ex **	In***	Ex	In	Ex	In	Ex	In
Yeast & Fungal (Log cfu)	Fresh	ND*	4.05	ND	2.74	ND	3.10	ND	3.05
	1	3.53	4.72	2.53	2.62	2.12	2.64	2.29	3.72
	2	3.65	4.78	2.28	2.33	2.11	2.61	2.25	3.17
	3	3.71	4.84	2.23	2.12	2.03	2.04	2.21	3.31
TBC**** (Log cfu)	Fresh	4.01	5.91	4.01	5.84	3.74	4.11	4.00	5.21
	1	5.12	6.01	3.77	5.77	3.21	3.87	3.84	5.54
	2	5.12	6.11	3.44	5.61	3.11	3.84	3.53	5.64
	3	5.00	6.11	3.12	5.57	2.51	3.71	3.11	5.60
Coliform bacteria		ND							

ND*: Not detected, Ex: Exterior, In***: Inside, TBC****: Total bacterial count**

Generally, control samples had higher counts of yeast & fungal as well as TBC in surface and middle of cheese than that of other treatments. The counts of yeast & fungal as well as TBC of the surface and middle of control samples increase with increasing of storage periods, while they decrease of cheese treated with Clove methanolic extract & sodium sorbate with increasing storage periods up to end of storage in all treatments. These results due to the inhibition effect Clove methanolic extract & sodium sorbate of growth yeast & fungal as well as TBC on surface and middle Ras cheese wheels during storage periods. These outcomes are consistent with those mentioned by **El-Fadaly *et al.* (2015)**. Moreover, TBC in surface and middle area of control samples during the ripening stage up to 3rd month were higher than those of other treatments of treated with extract Clove and sodium sorbate T_1, T_2 and T_3 in all treatments. In addition that, not all treatments revealed the presence of coliform bacteria from beginning of manufacture up to the end ripening stage 3rd month. Generally, the results showed that the sodium sorbate had higher antimicrobial effect than that with extract Clove on growth of yeast & fungal and general microorganism especially the responsible for ripening cheese during storage. On the other hand, the controlled hygienic conditions resulted in less fungal growth on Ras cheese wheels surface which inhibited by the extract Clove or sodium sorbate.

CONCLUSION

Our study reported that, the first in vitro test there was antibacterial activity of extract Clove on growing pathogens microorganism. Moreover, there were inhibition effect of surface and middle Ras cheese using Clove methanolic extract & sodium sorbate of growth yeast & fungal as well as TBC.

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النشاط المضاد للميكروبات لمستخلص القرنفل الميثانولي واختبار القوة الحافظة في الجبن الراس

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[2]قسم المحاصيل - كيمياء حيوية , كلية الزراعة , جامعة الازهر , فرع اسيوط, مصر.

الملخص

يهدف البحث الي دراسة تقييم النشاط الميكروبي لـ (مستخلص القرنفل الميثانولي) لبعض الميكروبات التي تنمو علي الجبن الراس بعد الصناعة وخلال فترة التسوية فترة ما قبل التسويق والتي تؤدي الي فساد المنتج وتردي الحالة الاقتصادية وايضا الاضرار بالحالة الصحية للمستهلك, ومقارنة النشاط المضاد لهذا المستخلص بأحد المركبات الكيميائية المستخدمة لهذا الغرض في هذا المنتج وهو(سوربات الصوديوم) داخل الحد المسموح به. قمنا أولا بدراسة مدي تأثير مستخلص القرنفل بتركيز (1جم/10ملجم) علي نمو نوعين من الفطريات هما(*Aspergillus flavus* , *Aspergillus Niger*), بالاضافة الي نوعين من البكتريا هما (*Salmonella , E. coli*) كان هناك تثبيط لنمو هذه الميكروبات مع اختلاف حجم التثبيط علي بيئة نموها داخل الاطباق وكان التركيز المستخدم هو الامثل لتثبيط هذه الميكروبات دون التأثير علي الفلورا الطبيعية والمسؤولة عن تسوية الجبن . قمنا في الخطوة الثانية بدراسة نفس التركيز من مستخلص القرنفل بالاضافة الي سوربات الصوديوم بتركيز 1.5جم/1كجم خثرة جبن بعد التعبئة وقبل عملية التشكيل للخثرة, فكان هناك ثلاث معاملات إضافة الي الكنترول علي النحو التالي: الكنترول (C) بدون أي اضافات و T_1 , T_2 باضافة مستخلص القرنفل والسوربات علي التوالي, وأخيرا T_3 طلاء المستخلص علي السطح الخارجي لقرص الجبن بشكل كامل بعد الانتهاء من التمليح الجاف مباشرة 12يوم. تم أخذ عينات لتقييمها ميكروبيولوجيا للجبن الطازجه قبل التخزين للتسوية وعلي 1, 2, 3أشهر من السطح ومن وسط القرص وقدر العد الكلي للبكتريا والفطريات والخمائر في كل العينات. أظهرت النتائج أنه لم يستدل علي بكتريا القولون في كل المعاملات وخلال فترات التخزين المختلفة, وجد أيضا تأثير للمادة الحافظة السوربات علي المحتوي الميكروبي عموما بشكل اكبر وخاصة المحتوي الميكروبي المسؤول عن تسوية الجبن مقارنة بتأثير مستخلص القرنفل الذي أعطي قدرة علي التثبيط دون التأثير علي عملية التسوية في المنتج بالاضافة الي أنه مصدر أمن صحيا دون أثار سلبية علي المستهلك.