HPLC ANALYSIS AND ANTIFUNGAL ACTIVITY OF SOME PLANT EXTRACTS AGAINST DECAY APPLE FRUITS

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ABSTRACT
This study was aimed to determine the qualitative and quantitative phenolic compounds. Results revealed that HPLC analysis identified Hydroxy derivatives of benzoic acids, hydroxy-cinnamic acids, in addition to flavonoids. The major compounds detected in clove were eugenol 32.5 mg/L, while in thyme the predominant phenol was gallic acid 35.9 mg/L. Quercetin was the greatest phenolic compound in both eucalyptus and sage 49.8 and 55.6 mg/L, respectively. Clove extract inhibited all selected pathogens entirely when applied at 20%. Subsequently, thyme extract at the same concentration inhibited the growth of *B. cinerea* completely. Eucalyptus extract revealed a lowest inhibition with 23.2%, 11.83%, and 7.83% after three, six, and nine days, respectively. *P. griseofulvum* showed remarkable susceptibility to extracts due to 51.99% growth inhibition followed by 25.33% and 20.67% for both *A. alternata* and *B. cinerea*, respectively.

Key words: Phytochemical analysis, clove, eucalyptus, sage, thyme, postharvest infection.

تحليل HPLC والفعالية التضادية الفطرية للمستخلصات النباتية ضد تعفن ثمار التفاح

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المستخلص
استهدفت الدراسة تحديد كمية و نوعية المركبات الفينولية. اظهرت نتائج تحليل HPLC تشخيص مشتقات هيدروكسيل حامض benzoic وكذلك مشتقات هيدروكسيل حامض cinnamic اضافة الى الفلافونيدات. ظهر eugenol كمكون رئيسي في القرنفل بمقدار ٣٢,٥ ملغم/مل بينما في الزعتر ظهر gallic acid هو الفينول السائد و بمقدار ٣٥,٩ ملغم/مل Quercetin. كان المكون الفينولي الاكثر في مستخلصات اليوكالبتوس و الميرمية وبمقدار ٤٩,٨و ٥٥,٦ ملغم/مل بالتتابع. ثبط مستخلص القرنفل جميع الممرضات المختبرة عند استخدامه بتركيز ٢٠٪ كما ان مستخلص الزعتر و بنفس التركيزثبط نمو الممرض *B.cinerea* بالكامل. اظهر مستخلص اليوكالبتوس اقل نسبة تثبيط للممرضات بالنسب ٢٣,٢٪،٨٣.١١٪، ٨٣.٧٪ بعد ثلاثة و ستة وتسعة ايام بالتتابع. بينت الدراسة الحساسية الفائقة للممرض *P. griseofulvum* للمستخلصات المدروسة حيث تثبطت نموها بنسبة ٥١,٩٩٪ وتليها *A. alternata* و *B. cinerea* حيث تثبط نموهما بنسب ٢٥,٣٣٪ و ٦٧.٢٦٪ بالتتابع.

الكلمات المفتاحية: تحليل الفايتوكيميائي، القرنفل، اليوكالبتوس، الميرمية، الزعتر، اصابات بعد الجني

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INTRODUCTION

Apple (*Malus domestica* Borkh) is the most fruit crop extensively in temperate regions (22). Recently, a problem of pre and postharvest fruit crops associated with core rot in major fruits has been encountered and studies have indicated several fungi causing moldy core or decay of dropped and full ripen fruits on the tree and during storage. Thus, postharvest losses of apple fruits are a serious problem, particularly of fungal pathogens that are mainly responsible for postharvest spoilage of about 5 to 25% of apples and pears (3, 16, 20). *A.alternata, B. cinerea, and Penicillium spp.* particularly of *P. expansum* and *P. griseofulvum* were the predominant pathogens responsible for the main economic losses of apple decay in different regions of the world despite using modern storage facilities(10, 29, 30). The practices for controlling these different rotting agents consist essentially of applying synthetic fungicides during the pre-harvest period or immediately after harvest. However, this controlled practice is currently being challenged because of the emergence of pathogen strains resistant to the main registered active ingredients and because of chemical residues that remain in the fruit that greatly harm human health and the environment (7). Hence, several alternative methods use such as plant extracts and essential oils that produce a wide variety of secondary metabolites in response (13,26, 28), they can easily decompose, are nature friendly, and are nontoxic. Extracts and essential oils contain secondary toxic compounds metabolized by plants and stored in the plant cell vacuoles, among them are phenolic, steroid, and terpenoid compounds (34). Their concentrations depend on the environmental conditions and the incidence of the pathogen, which give them bio-pesticidal characteristics. Furthermore, they are biodegradable (5, 24, 27, 31). Consequently, these compounds could be well integrated with the biological control of plants diseases. Antifungal effect of essential oils and extracts of thyme and eucalyptus evaluated in Iran against *A. alternata* on potato (12). Thirteen species of such medicinal plants as thyme and cumin were studied against apple mold caused by *B. cinerea* (4). High Performance Liquid Chromatography HPLC analysis indicated that polyphenolic compounds, such as phenolic acids and flavonoids are important constituents in many plants, their identification and quantification can give vital information about the antioxidant function, food quality, and health benefits. Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acid, the most common hydroxybenzoic acid are benzoic, vanillic, gallic, and protocatechuic acids which are mainly present in the form of glucosides in foods. The most common forms of hydroxycinnamic acid are coumaric, caffeic, and ferulic acids. Chlorogenic acid is the most familiar one (13, 32, 39). The main objectives of the present work were to screen phytochemical analysis of phenolic acids and flavonoids using HPLC and evaluate the antifungal activity of clove buds, eucalyptus leaves, sage, and thyme foliage extracts against several pathogens of postharvest apple decay.

MATERIALS AND METHODS

Collection of plant materials and preparation of plant extracts: Different plant samples of clove (*Syzygium aromaticum* L.M.Perry), eucalyptus (*Eucalyptus globulus* Labill.), sage (*Salvia officinalis* L.), and thyme (*Thymus vulgaris* L.) were collected from the College of Agricultural Engineering Sciences, Duhok mountains, and local markets. The plant extracts were prepared as follows: After washing under running tap water and sterile distilled water, the sterilized materials were wrapped with two-fold newspapers and dried in the shad. One gram of grounded plant parts (clove buds, eucalyptus leaves, sage, and thyme foliage) was mixed with 100ml sterile water. The extracts are firstly filtered through muslin cloth and then through whatman filter papers. Later, the extracts were used as a standard solution of %100 concentration.

HPLC analysis of phenolic and flavonoid compounds: Standards of phenolic acids (gallic, chlorogenic, ellagic, ferulic, sinapinic, and vanillic) and flavonoids of (catechin, epicatechin, eugenol, isorhamnetin, kaempferol, luteolin, quercetin, and rutin) compounds. All these chemicals were provided by (Sigma-Aldrich, Co., Germany). Quantification of individual phenolic

compounds of selected plant samples was conducted by reversed-phase HPLC model (SYKAMN HPLC chromatographic system, Germany) equipped with a Zorbax Eclipse Plus-C18-OSD column (250 X 4.6mm, 5µm), the column temperature was 30ºC., pump model: S2100 Quaternary Gradient Pump. The mobile phase for eucalyptus, sage, and thyme extraction was composed of methanol (solvent A) and1% formic acid in water (v/v) (solvent B) and performed as follows: initial 0-4 min. 40 % B; 4-10 min. 50 % B; and flow rate of 0.7ml /min. Detection of the phenolic compound was carried out with a UV visible detector at 280 nm. The volume of samples was 100 µl and standards 100 µl injected using an auto-sampler. For clove extract, the mobile phase composed of 95% acetonitrile + 0.01% Triflouroacetic acid (solvent A) and 5% acetonitrile + 0.01% Triflouroacetic acid (solvent B) at flow rate of 1 ml/min. The gradient program was: 10% A from 0–5 min; 25% A from 5-7 min; 40% A from 7–13 min. Detection of phenolic compounds was carried out with a UV-visible detector at 278 nm.

Antifungal activity of plant extracts: In vitro, the inhibition effects of botanical extracts were assessed against the pathogens of *Alternaria alternata* (Fr.Keissl*.), Botrytis cinerea* (Pers**.)**, and *Penicillium griseofulvum* **(**Dierckx) using poisoning food technique as described by Mohana and Raveesha (25) as follows: PDA medium was prepared and amended with different concentration of botanicals viz. 5, 10, 15, 20%. Mycelial discs of selected pathogens were placed at the center petri plates and incubated at 25°C± 2°C for nine days. The plates without formulation served as control. The radial mycelial growth (mm) was measured. All experiments were carried out in triplicates and percent reduction of mycelia growth over control was calculated after 3, 6, and 9 days according to the following formula:

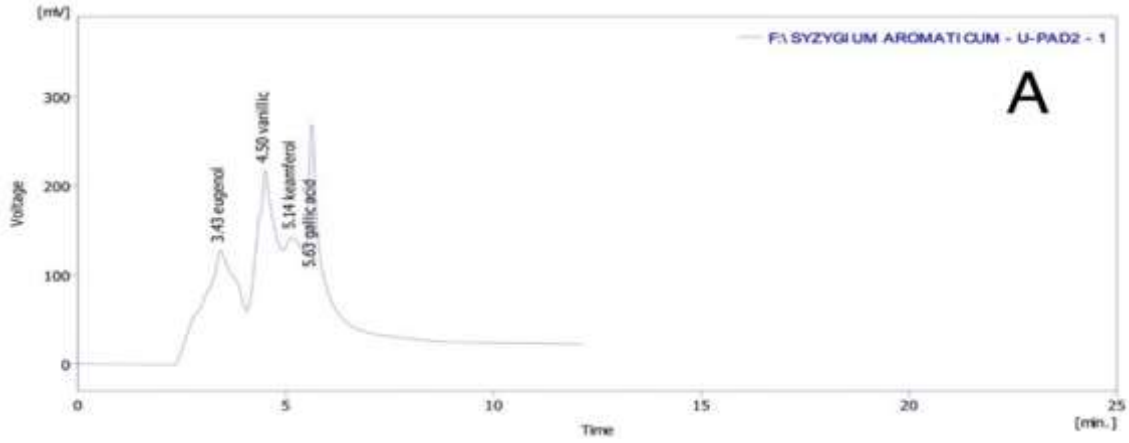
% inhibition = [(DC – DT) / DC] X 100

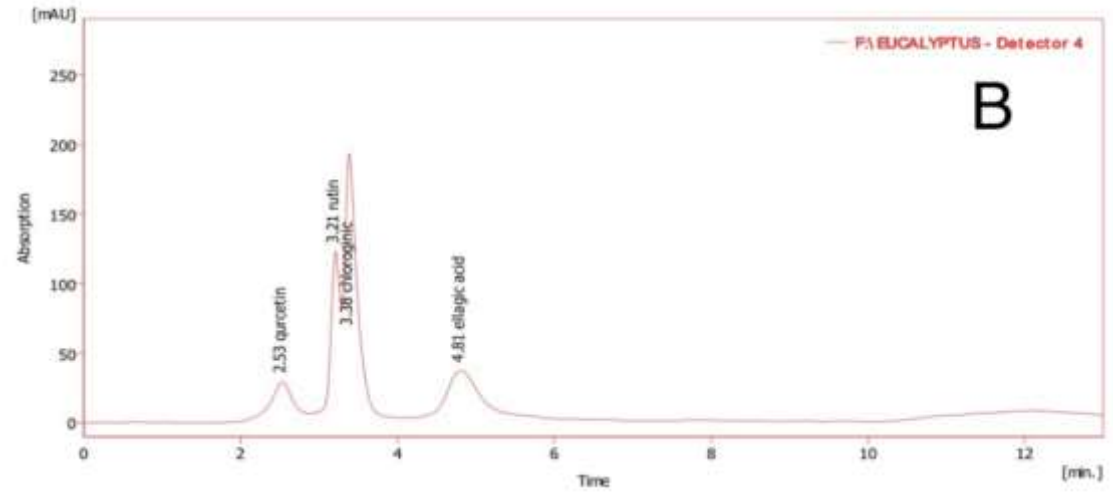
Where: DC = Average increase in mycelial growth in control plate DT = Average increase in mycelial growth in treatment plate. The data were analyzed using the software SAS, Ver. 9.4. (SAS Institute Inc., 2016). Data were subjected to analysis of variance (ANOVA) and means of the treatments were compared by Duncan Multiple Range Test at $P \leq 0.05$.

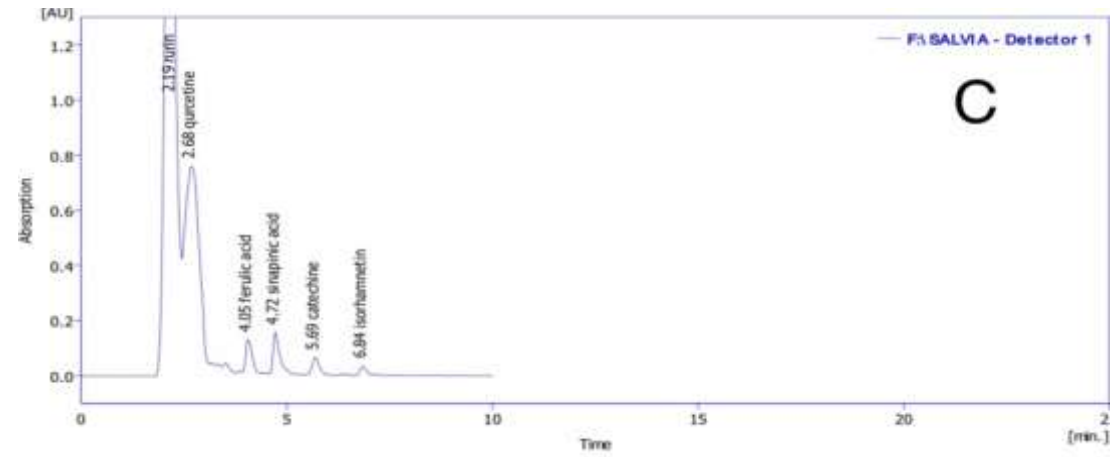
RESULTS AND DISCUSSION

Phytochemical composition of crude aqueous extracts: HPLC analysis was employed to define qualitative and quantitative content of phenols and flavonoids in investigated extracts viz clove, eucalyptus, sage, and thyme. Compounds of each sample were identified by comparing their relative retention times (Rt) and online ultraviolet (UV) spectra with those of the chromatogram of the standard. The concentration of an individual compound was calculated based on peak area measurement as shown in Fig. (1) and included hydroxy derivatives of benzoic acids (gallic, ellagic, and vanillic) and hydroxy derivatives of cinnamic acids (chlorogenic, ferulic, and sinapinic) in addition to flavonoids (catechin, epicatechin, eugenol, isorhamnetin, kaempferol, luteolin, quercetin, and rutin). Arif et al. (2) defined phenolic compounds are one of the major families of secondary metabolites in plants, they are about 10.000 individual polyphenols are very important for plants to contribute to resistance to pathogenic microorganisms. Table (1) shows that eugenol in clove extract was found as a major component with 32.5 mg/L followed by gallic acid with 24.8 mg/L, kaempferol with 12.8 mg/L, and vanillic acid 9.8 mg/L. Investigation of clove extract by Karm (14) proved that clove extract content of eugenol was the best antimicrobial agent, particularly with the high doses. In this aspect, Lee and Shibamoto (18) reported that the whole essential oil of clove or its main extract component is eugenol C6H12O2; 4-ally 1-2-methoxy phenol). While gallic acid had the second level in clove, it was the predominantly identified phenol in thyme with 35.9 mg/L followed by epicatechin 29.8 mg/L and catechin 26.9 mg/L. These results were in agreement with Koksal et al. (15) when identified the total phenolics of gallic acid and flavonoids of catechin in thyme extract. Despite quercetin being the greatest phenols in both eucalyptus and sage extracts with 49.8 and 55.6 mg/L, it was absent in both clove and thyme. Dezsi et al. (8) determined the main flavonoid and polyphenols in eucalyptus are hyperoside (quercetin), rutin, and ellagic acid.

Subsequently, the rutin component present with the second level in both eucalyptus and sage with 34.8 and 42.9 mg/L, respectively. The results revealed that the greatest number of phenolic acids and flavonoids are assets in both extracts of sage and thyme, and the maximum concentration of these compounds was quercetin with 55.6 mg/L and gallic acids 35.9 mg/L in both extracts, respectively. Works of the literature confirmed that sage extract contains polyphenols of ellagic acids, rutin, chlorogenic acid, quercetin, and catechin (19, 23, 38). In this aspect, Sarhan et al. (35) reported that HPLC analysis of a methanolic extract of thyme and sage showed the presence of caffeic acid, cinnamic acid, chlorogenic acid, and some flavonoids of luteolin and quercetin, and thyme extract possessed the best antioxidative activities. However total phenol compounds and flavonoids were identified in thyme 154.5 mg/L, followed by sage 151.6 mg/L, Eucalyptus 115.1 mg/L, and clove 79.9 mg/L.







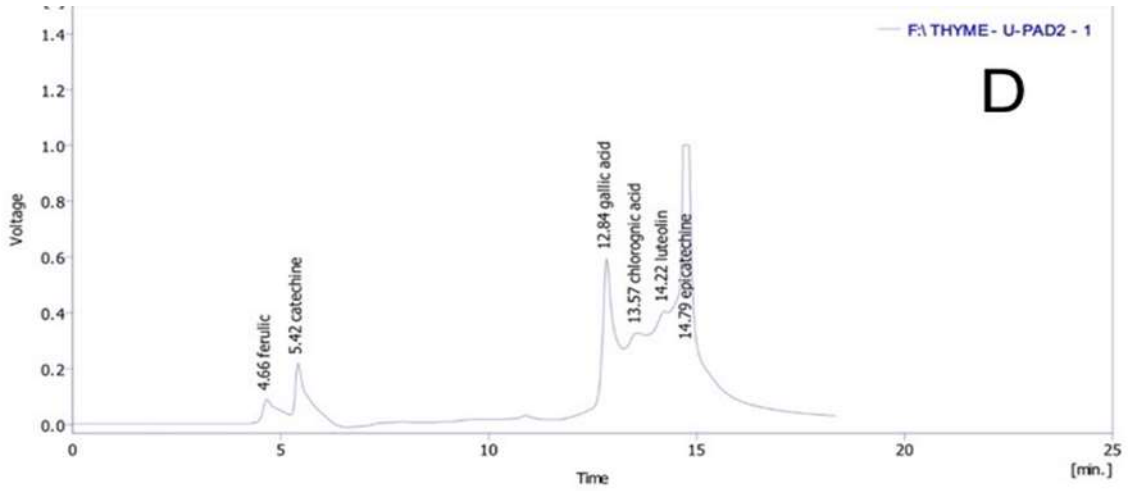


Figure 1. HPLC chromatogram of aqueous extract and Uv spectra of identified phenolic and flavonoid compounds in clove (A), eucalyptus (B), sage (C), and thyme (D).

Table 1. Polyphenol compounds determined by HPLC in crude aqueous extracts

phenolic Compounds	Clove		Eucalyptus		Sage		Thyme	
	R.t [min]	mg/L	R.t [min]	mg/L	R.t [min]	mg/L	R.t [min]	mg/L
Catechin	-	-	-	-	5.690	12.1	5.420	26.9
Chlorogenic Acid	-	-	3.383	14.7	-	-	13.572	20.4
Ellagic Acid	-	-	4.810	15.8	-	-	-	-
Epicatechine	-	-	-	-	-	-	14.788	29.8
Eugenol	3.432	32.5	-	-	-	-	-	-
Ferulic Acid	-	-	-	-	4.053	18.9	4.664	22.6
Gallic Acid	5.628	24.8	-	-	-	-	12.840	35.9
Isorhamnetin	-	-	-	-	6.843	8.2	-	-
Kaempferol	4.504	12.8	-	-	-	-	-	-
Luteolin	-	-	-	-	-	-	14.224	18.9
Guercetin	-	-	2.533	49.8	2.680	55.6	-	-
Rutin	-	-	3.210	34.8	2.190	42.9	-	-
Sinapinic Acid	-	-	-	-	4.720	13.9	-	-
Vanillic Acid	4.504	9.8	-	-	-	-	-	-
Total	-	79.9	-	115.1	-	151.6	-	154.5

In vitro: antifungal activity of plant extracts on the *A. alternat, B. cinerea,* and *P. griseofulvum* mycelial growth: The results in Table (2) show that the inhibition percentage of pathogens growth was the most prominent after three days of incubation compared to six and nine days. Clove extract in particular inhibited all evaluated pathogens entirely after three days when applied at 15 and 20%.

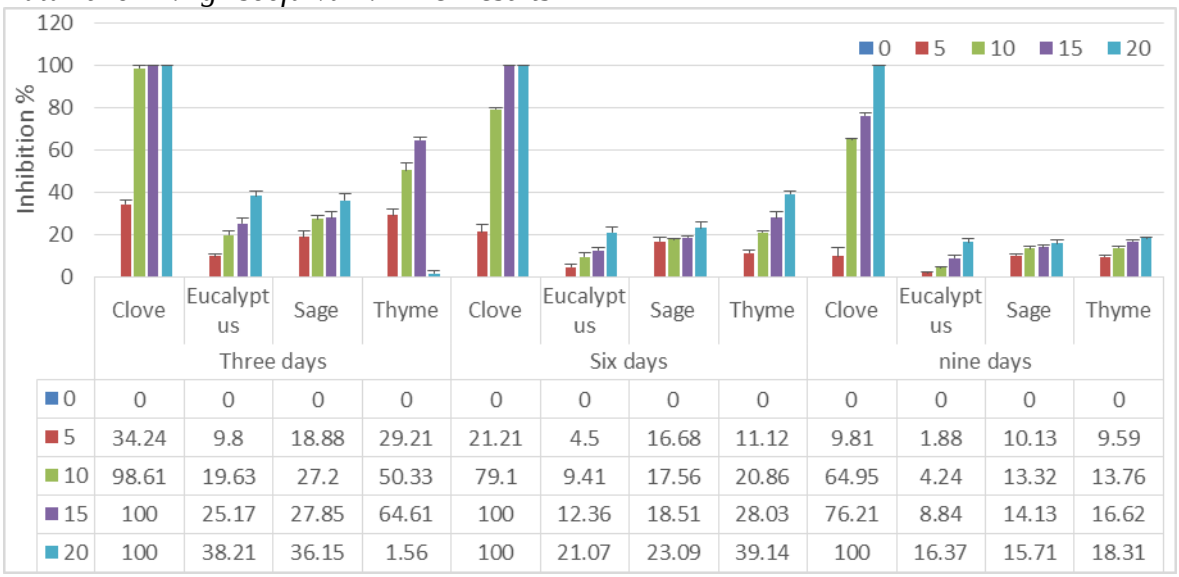
Subsequently, thyme extract at the same mentioned concentration inhibited the growth of *B. cinerea* completely (Plate 1). Furthermore, the effective mean of the clove extract was apparently and exceeded up to 83 % after three days, and persisted even after six and nine days when attained pathogens inhibition to 75.08 %, and 62.74 %, respectively.

Table 2. Antifungal activity of plant extracts on inhibition of pathogen's growth in vitro.

Plant extracts	% conc.	%Radial growth inhibition											
		3days				6days				9 days			
		A. alternata	*B. cinerea*	*P. griseofulvum*	*Mean*	*A. alternata*	*B. cinerea*	*P. griseofulvum*	*Mean*	*A. alternata*	*B. cinerea*	*P. griseofulvum*	Mean
Clove	5	15.07 l-p	32.79 i	54.85 efg	83.21 a	11.16 nop	0 r	52.48 ef	75.08	0 j	0 j	29.42 g	62.74 a
	10	95.82 a	100 a	100 a		51.63 fg	100 a	85.66 b	a	26.27 g	100 a	68.58 c	
	15	100 a	100 a	100 a		100 a	100 a	100 a		40 f	100 a	88.62 b	
	20	100 a	100 a	100 a		100 a	100 a	100 a		100 a	100 a	100 a	
Eucalyptus	5	12.03 op	3.27 q	14.09 m-p	23.2 d	6.39 pqr	0 r	7.15 o-r	11.83	0 j	0 j	5.63 i	7.83 d
	10	14.03 m-p	26.79 ij	18.09 k-o		9.65 n-q	0 r	18.59 lm	d	0 j	0 j	12.71 h	
	15	17.23 k-o	44.23353	24.03 jk		10.58 n-q	0 r	26.51 jk		0 j	0 j	26.51 g	
	20	21.11 j-n	47.93 h	45.60 h		14.75 mn	0 r	48.45 fg		0 j	0 j	49.11 e	
Sage	5	13.28 nop	12.13 op	8.03 pq	27.52 c	7.25 o-r	0 r	38.39 i	18.96	0 j	0 j	42.40 f	13.32 c
	10	21.80 j-m	26.79 ij	9.69 opq		11.64 nop	0 r	41.46 hi	c	0 j	0 j	30.39 g	
	15	22.52 jkl	51.34 fgh	27.27 ij		14.05 mno	0 r	45.43 gh		0 j	0 j	39.95 f	
	20	27.61 ij	53.56 efg	56.21 ef		22.13 kl	0 r	47.14 fgh		0 j	0 j	47.14 e	
Thyme	5	17.32 k-o	26.67 ij	43.64 h	53.25 b	3.6 qr	0 r	29.721 j	24.79	0 j	0 j	28.76 g	14.57 b
	10	23.69 jk	66.11 cd	61.21 de		4.11 qr	0 r	58.47 de	b	0 j	0 j	41.28 f	
	15	24.44 jk	100 a	69.39 c		10.16 n-q	14.31 mn	59.62 d		0 j	0 j	49.88 e	
	20	27.54 ij	100 a	78.94 b		28.13 jk	16.47 lmn	72.81 c		0 j	0 j	54.94 d	
Mean		34.59 c	55.73 a	50.07 b		25.33 b	20.67 c	51.99 a		10.39 c	18.75 b	44.71 a	

*** Within each independent factor and interaction means followed by the same letters aren't significantly different according to Duncan's Multiple Range Test ($p \leq 0.05$). Each value of interaction is the mean of three replicates**

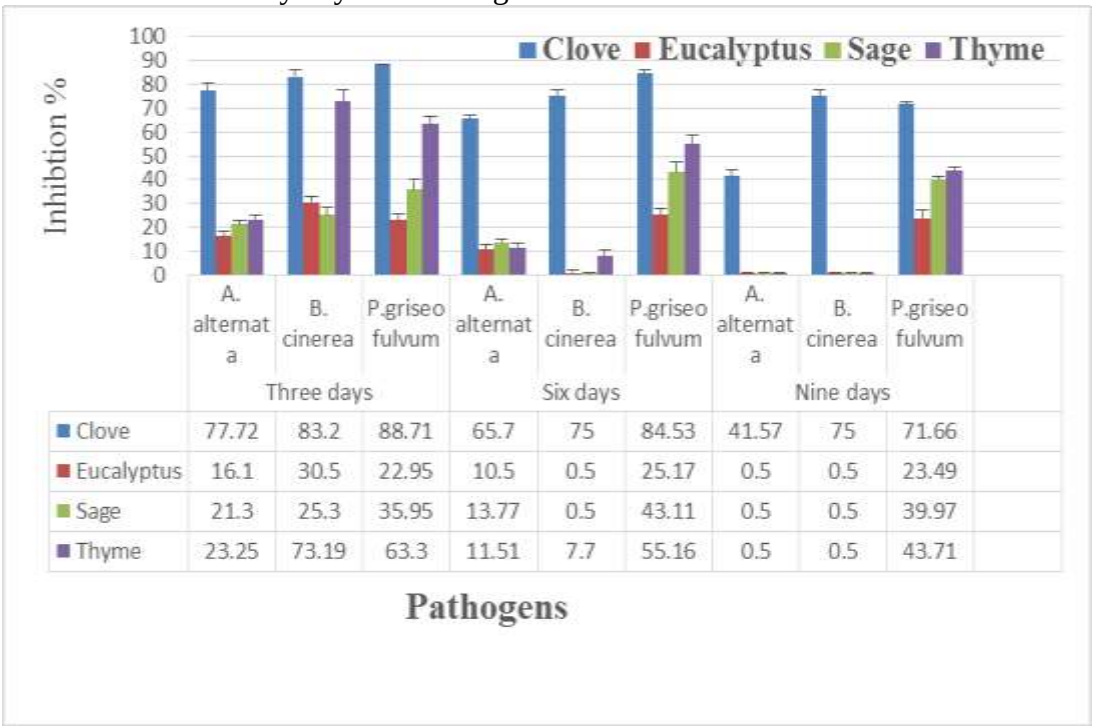
The presence of selected pathogens on the synthetic media of PDA amended with extracts of clove and thyme resulted in arise in redox enzymes of polyphenols oxidase and peroxidase activity that play a defense role in prevent microorganisms from attacking through oxidation of natural phenols identified by HPLC to produce quinones which undergo a series of polymerization reactions leading to the production of melanin that possess antimicrobial activities (9) and other biotic stress (33). After six days, clove extract at 15 and 20% continued its effective against selected pathogens and inhibited their wholly growth. Thus, the effective mean of this extract exhibited considerable efficiency with 75.08% compared to 24.79%, 18.96% and 11.82% for thyme, sage, and eucalyptus extracts, respectively. In return, *P.griseofulvum* showed noticeable susceptibility to extracts due to 51.99% growth inhibition followed by 25.33%, and 20.67% for both *A. alternata* and *B. cinerea,* respectively. These results coincided with (36) when revealed the efficacy of 20 plant species against Penicillium species on apple. Contrariwise, no inhibition found for *B. cinerea* on both extracts of eucalyptus and sage regardless of their concentrations. After nine days, the most concentrations of thyme, sage, and eucalyptus failed in depression of both *A. alternata, B. cinerea*. Vice versa clove extract at 20% shows entire inhibition for *A. alternata* and *P. grisoefulvum*. The results encouraged by (17) that found the clove extract to be very active against the tested *penicillium* spp. due to its content of eugenol that possess high activity against microorganisms. Hence, *P.griseofulvum* was more susceptible to phenolics content of clove and thyme. *B. cinerea* was also completely inhibited on clove extract at 10, 15, and 20%. Therefore, the mean activity of clove reached to 62.74% compared to 14.57 %, 13.32%, and 7.83% for thyme, sage, and eucalyptus, respectively. It was reported that the effect of clove and thyme extract as alternative synthetic bio-fungicides in controlling of *P. digtatum* on postharvest apple fruits (21). Results in Fig. (2) present the interaction between extracts and their concentrations. Clove extract at 15%and 20% resulted in full inhibition after three and six, days. This extract continued its activity even after nine days. The poor extract effectiveness resulted by eucalyptus at 5%,10%, and 15% which was non-significant with control. In general, the fungal activity of examined extracts increased according to increasing their concentration particularly of clove. Adaramola and Onigbinde (1) illustrated that clove buds have more flavonoids and phenols than tannin and terpenes with a positive correlation among all active compounds of clove buds and their antimicrobial and antioxidant capacity due to the presence of many phytochemicals and their synergistic effects.



	Three days				Six days				nine days			
	Clove	Eucalyptus	Sage	Thyme	Clove	Eucalyptus	Sage	Thyme	Clove	Eucalyptus	Sage	Thyme
0	0	0	0	0	0	0	0	0	0	0	0	0
5	34.24	9.8	18.88	29.21	21.21	4.5	16.68	11.12	9.81	1.88	10.13	9.59
10	98.61	19.63	27.2	50.33	79.1	9.41	17.56	20.86	64.95	4.24	13.32	13.76
15	100	25.17	27.85	64.61	100	12.36	18.51	28.03	76.21	8.84	14.13	16.62
20	100	38.21	36.15	1.56	100	21.07	23.09	39.14	100	16.37	15.71	18.31

Figure 2. Effect of interaction between extracts and their concentrations on pathogens growth after three, six, nine days

Fig. (3) clarified the interaction effect between extracts and pathogens, it was clear that *P. griseofulvum* exhibited high sensitivity to clove extract since inhibited its growth by 88.71%, 84.53% and 71.66% after the duration of three, six, and nine days, respectively. Clove extract also gave a similar effect of toxicity with 83.2% against *B. cinerea* after three days, and no inhibition was shown for this pathogen when grown on sage and eucalyptus even after six days. High levels of eugenol contained in clove are responsible for strong antimicrobial activity by denaturing proteins and reacting with cell membrane phospholipids affect their permeability (6). Thyme, sage, and eucalyptus failed in an expression of *A. alternata* and B. *cinerea* after nine days. Phenols are the active antifungal compounds in most plant extracts (37). Many potential modes of action by phenol against pathogens are based on impairment of enzymatic processes including energy production and weakening or destroying the permeability berries of the cell membrane or affecting the synthesis of nucleic acids (11).

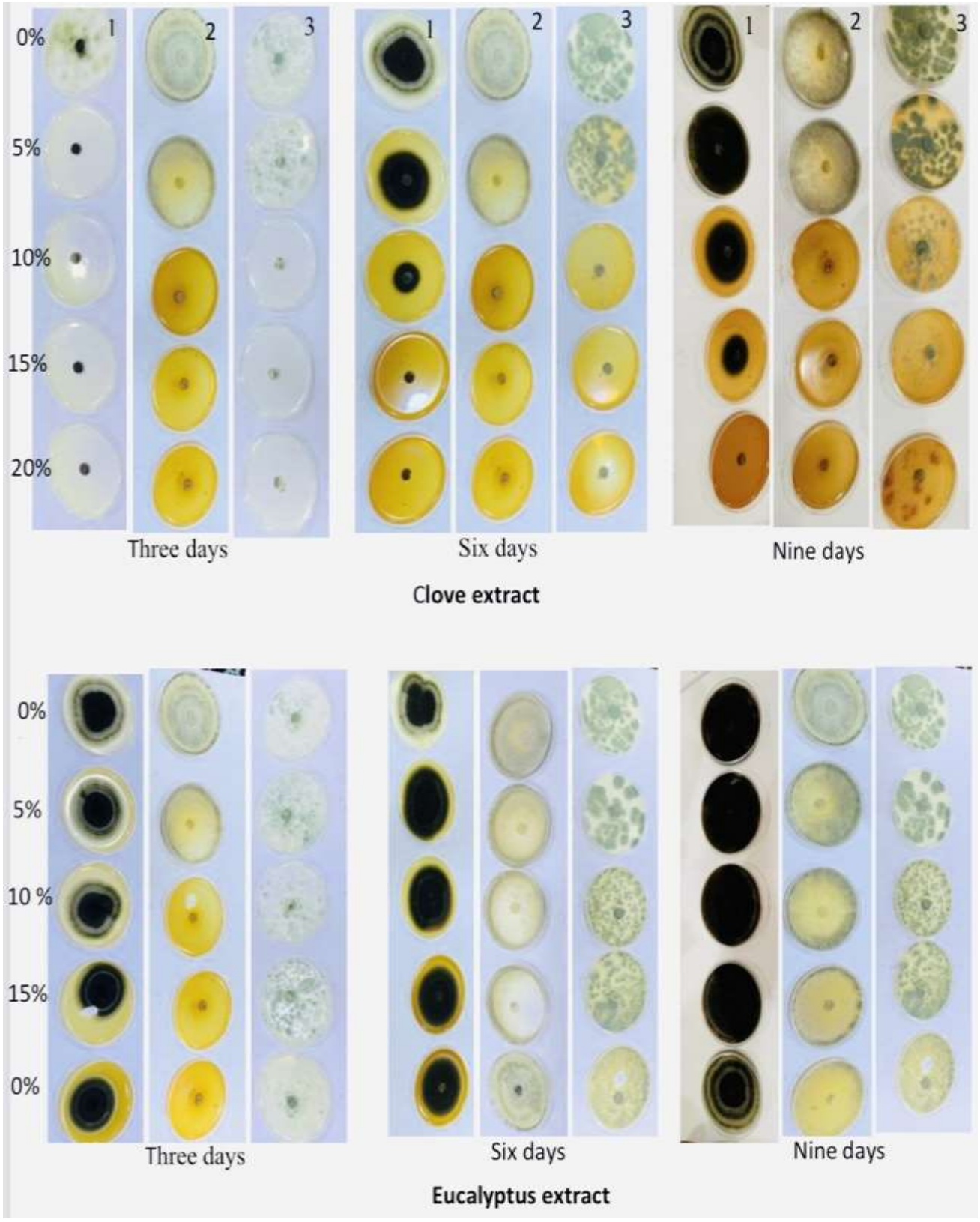


	A. alternata	B. cinerea	P.griseofulvum	A. alternata	B. cinerea	P.griseofulvum	A. alternata	B. cinerea	P.griseofulvum
	Three days			Six days			Nine days		
Clove	77.72	83.2	88.71	65.7	75	84.53	41.57	75	71.66
Eucalyptus	16.1	30.5	22.95	10.5	0.5	25.17	0.5	0.5	23.49
Sage	21.3	25.3	35.95	13.77	0.5	43.11	0.5	0.5	39.97
Thyme	23.25	73.19	63.3	11.51	7.7	55.16	0.5	0.5	43.71

Figure 3. Effect of interaction between plant extracts and pathogens on their growth inhibition after three, six, nine days

CONCLUSION

These results demonstrate that HPLC analysis of aqueous extracts of clove (*Syzygium aromaticum*), eucalyptus (*Eucalyptus globulus*), sage (*Salvia officinalis*) and thyme (*Thymus vulgaris*) identified several phenolic acids and flavonoids particularly of eugenol in clove, gallic acids in thyme, and quercetin in sage and eucalyptus. Functional extracts could provide an alternative method of using hazardous chemical fungicides for treatment of apple fruits during harvest, storage, and transportation to reduce postharvest infections caused *A. alternata*, *B. cinerea* and *P. griseofulvum*, the major causes of apple decay, since investigation antifungal activity of these extracts against apple rot pathogens confirmed effective and remarkable inhibition of their mycelial growth with considerable potency of a natural preservative to avoidance apple from phytopathogenic fungi, and may also prevent the spoilage of other food commodities during storage with low toxicity and don't persist in the environment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Three days
Six days
Nine days
Eucalyptus extract

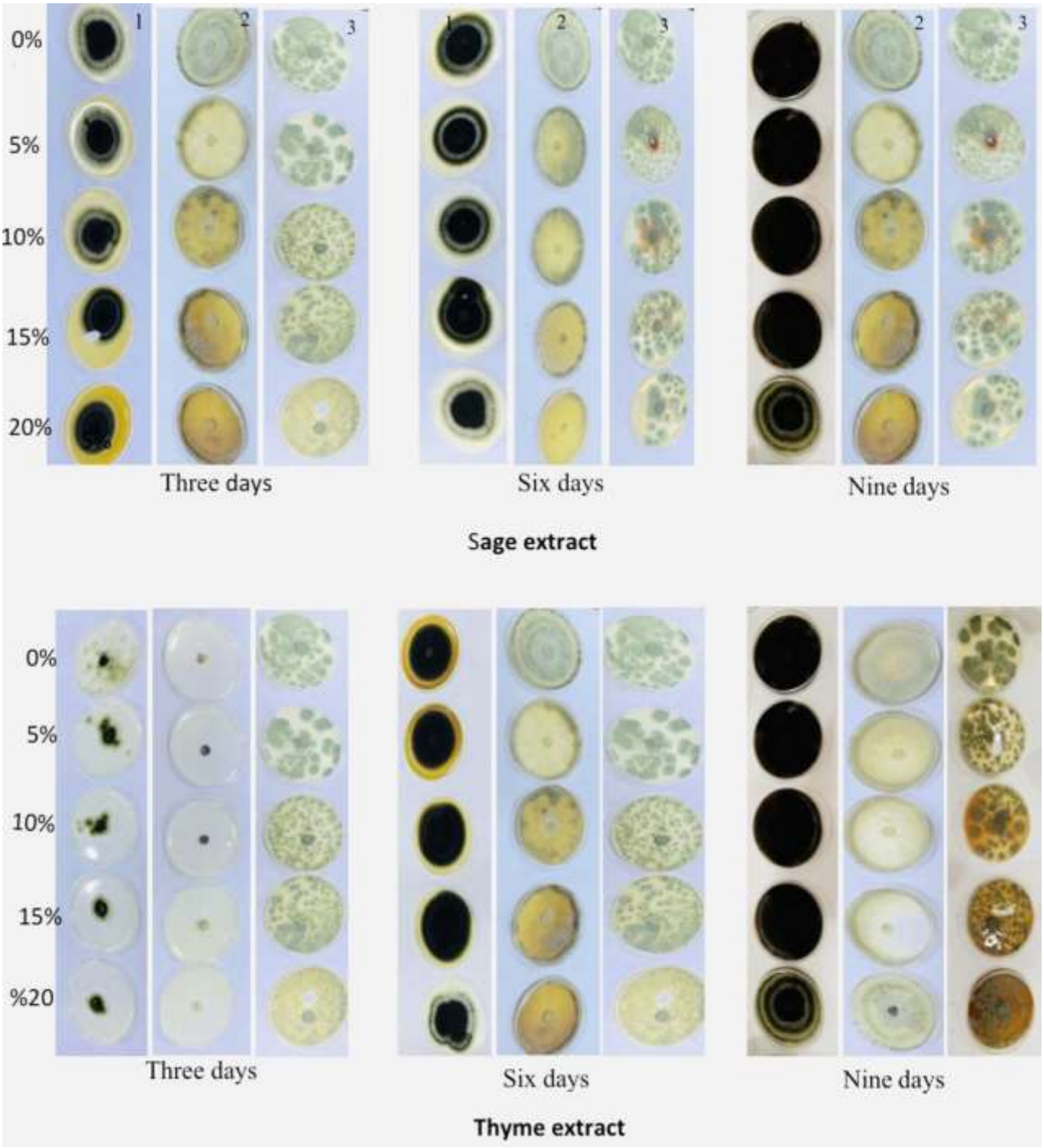

Plate 1 In vitro antifungal activity of plant extracts (clove, eucalyptus, sage, and thyme) on growth inhibition for 1. *A. alternata*, 2. *B. cinerea*, and 3. *P. griseofulvum*

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