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ANTIFUNGAL EFFECT OF SPICE EXTRACTS - POSSIBLE SOLUTIONS FOR BIOLOGICAL PRESERVATION OF FOOD

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Abstract: *Aromatic plants used as spices are effective sources for food additives, used both as taste correctors and for the purpose of preserving food. The paper aims at identifying aromatic herbal extracts that summarize several qualities: to help improve the taste and olfactory qualities of food, to stimulate digestive secretions by facilitating digestion and, last but not least, to biologically preserve food, contributing to the reduction in the number and amount of synthetic additives. For this purpose, aqueous extracts of Cinnamomum zeylanicum ritidom, Laurus nobilis leaves and Eugenia caryophyllata floral buds, from commercial sources, were prepared. The extracts were tested on saprophytic fungi cultures, which usually infest food by causing alteration and they were obtained using the SER 148 extractor. The Aspergillus, Mucor and Penicillium cultures were made by selection from environment. The extracts of the three spice species have demonstrated significant fungal activity, inhibiting mold growth. The most powerful effect is recorded by the cloves extract, Eugenia caryophyllata, followed by the cinnamon extract. Having in view the results of these experimental studies we consider that spice extracts can be used in the medium term food storage, as they reduce the amount of synthetic preservatives and replace them by natural products.*

Keywords: *biological methods, mold, vegetal extracts.*

1. Introduction

Fungal colonies are spread throughout the environment. They have an extensive capacity to colonize organic materials, soils, leaves and wood, but also food, textiles, paper, archives, museums and libraries, generating their degradation. It generates significant damage. Their presence in food is particularly harmful due to aflatoxins, with carcinogenic potential. Long-term storage of food in safe conditions, using as few as possible preservatives, is an objective of the current food industry. The aromatic plant species used as spices owe their digestive effect to essential compounds such as monoterpenoids and sesquiterpenoids. These substances are also known to have inhibitory effects on the development of both microbial and tumor cells. More recently, a novel antimicrobial peptide namely "Plantaricin CS" with a wide antibacterial activity was isolated from coriander leaf extract and the greatest antimicrobial effect of it was shown on *S. aureus* strain but also against *Fusarium* and *Aspergillus* species [1- 13]. In recent time, a new antimicrobial peptide namely "Plantaricin CS" was isolated from coriander leaf extract and has shown a highly effective antifungal activity against *Penicillium lilacinum* (MIC = 2.5 mg/mL) and *A. niger* (MIC = 2.3 mg/mL) [13].

Exotic spices, as well as aromatic plants in the European spontaneous flora, are tested for their popular properties, including the ability to inhibit mold growth [14]. Components of these plants, such as essential oils and other substances are responsible for germicidal effects. The allelopathic effect can be extended to yeast strains and bacteria, highlighting these plants as potential alternatives to synthetic antibiotics. Plants from the local flora might present a new alternative source for possible bioactive substances. The culinary herbs and spices have major advantages being inexpensive, safe (used since generations), and easily accessible. Nevertheless, fractionation, purification, and isolation processes are underway with the aim to isolate and chemically modify bioactive natural compounds [15-17].

The antifungal effect of spice extracts is not limited to preserving food. It can be used in the preservation of any organic material likely to be degraded by molds, such as textiles, art objects, and construction materials. The purpose of using plant extracts can be also the reduction of synthetic fungicides, polluting to the natural environment [4, 18-20]. Most of the prevalent synthetic preservatives have multiple side effects to the health and environment. In this context, plant essential oils which have been used in traditional medicine and pharmaceutical preparations are gaining interest by the food industries for the development of eco-friendly food preservatives with functional properties [21].

2. Matherials and methods

Isolation of fungi from local soil was performed on Dichloran 18% Glycerol (DG18) Agar (Merck, Darmstadt) and Dichloran Rose Bengal Chloramphenicol (DRBC) Agar (Merck, Darmstadt). Under aseptic conditions, 20 g of soil was homogenized in 180 mL of sterile peptone water (0.1 g of peptone/100 mL of distilled water). After this, samples were shaken for 10 min at 200 rpm [22]. One milliliter of the obtained stock solution was transferred into a Petri plate (Ø 9 cm), in which the medium was poured and samples were incubated for 7 days at 25 ± 2 C. In order to obtain pure cultures and perform the identification, colonies that were assumed to belong to the *Aspergillus, Mucor* and *Penicillium genus* (according to the macromorphological characteristics) were re-inoculated to the Czapek Yeast Autolysate Agar, CYA ($NaNO_3$ 3 g; K_2HPO_4 1 g; KCl 0.5 g; $MgSO_4 \circ 7H_2O$ 0.5 g; $FeSO_4 7H_2O$ 0.01 g; yeast extract 5 g; sucrose 30 g; agar 20 g; distilled water 1000 mL). After this, samples were incubated for 7-14 days at 25 ± 2 C. The isolates assumed to belong to the genus *Aspergillus* and *Mucor* were inoculated to the Csapek-Dox Agar, in order to obtain monosporic cultures. Monosporic cultures were incubated for 10 and 14 days under a cyclic regime-mode with 12 h of combined light (fluorescent light and NUV e near ultraviolet) and 12 h of darkness at 25 C in order to stimulate the formation of conidiogenous structures. Obtained pure cultures of fungi were identified according to the keys for determination (colony diameter, color and texture; microscopic characteristics e hyphae and conidiophore appearance, size and shape of vesicles, metulae, phialides and conidia). Seven-day fungal cultures grown on Czapek-Dox Agar were used for preparation of the fungal spore suspension tests. The spores were harvested with sterile loop in 10 mL of medium which contained 0.5 mL/100 mL Tween 80 and 0.2 g/100 mL agar in sterile distilled water and aseptically transferred into sterile test tubes. The spore suspensions were adjusted with the same solution to give a final spore concentration of 10^6 spores / mL by using the

hemocytometer. Csapek-Dox was the medium used for antifungal investigations. Csapek-Dox was utilised in the following variants:

V1- Csapek-Dox was poured in sterile Petri plate (Ø 9 cm), 12 mL into each plate. Plates were centrally inoculated by spotting the 1 mL of spore suspension (10^6 spores/mL) in the middle of the plate using an inoculation needle to give a circular inoculum of approximately 2 mm in diameter (one inoculum per plate). After inoculation, the Petri plates were closed with a parafilm and incubated at 25 ± 2 C for 5 days. The filter paper rings, 6 mm in diameter, were soaked in the dilute extracts as follows: V1.1: 1:2; V1.2: 1:5, V1.3: 1:10. Paper loops have been applied to fungal crops. After 24 and 48 hours of incubation, the inhibition area around the rounds is measured.

V2 Csapek-Dox was divided into equal volumes (10 ml), poured into Erlenmeyer (50 ml) flasks and autoclaved at 121 C for 15 min and then cooled to 45 C. Each of the extracts were added in the liquid medium at following concentration: V2.1 – 1 ml extract in 10 ml medium, V2.2. 2 ml extract in 10 ml medium, V2.3 3 ml of extract in 10 ml medium, V2.4 without vegetal extract. After inoculation, the Erlenmeyer were closed with a parafilm, incubated and shaken at 200 rpm, at 25 ± 2 C for 3 days.

V3 Csapek-Dox medium was poured in sterile Petri plate (Ø 9 cm), 10 ml into each plate. In each plate was mixed one extract in following variants: V3.1 1 ml extract mixed with 10 ml medium, V3.2 – 2 ml extract in 10 ml mediu, V3.3 3 ml extract in 10 ml medium and V3.4. without vegetal extract.

V4 – Csapek-Dox medium was poured in sterile Petri plate (Ø 9 cm), 10 ml into each plate. In all tested (for each extract concentration), plates were inoculated 1 ml of spore suspension (10^3 spores/ml) in zig –zag modality of entire surface of the plate, using an inoculation needle. Before spores inoculated, undiluted extracts were applicated on the half of developed colonies, and the other half was preserved as witness.

The effect of the each extracts on the growth of fungi and fungal count were carried out in 3 replications.

Extracts.

Ripen and dried fruits of *Carum carvi, Coriandrum sativum* and *Anethum graveolens* were obtained by commercial sources, washed with tap water followed by distilled water to remove the dust particles. The fruits were allowed to dry at room temperature (37 ^{0}C). Hundered grams of fruits were crushed and soaked in 100 ml of double distilled water. The mixtures of water and seeds were submitted for 1h to water distillation using a SER 148-type apparatus to produce an hidroextraction.

Also for utilised spices: twenty grams of: *Cinnamomum zeylanicum* barks, *Laurus nobilis* leaves, *Eugenia caryophyllata* flower buds from local market were soaked in 100 ml of double distilled water. The mixtures of water and spices were submitted for 1h to water distillation using a SER 148-type apparatus to produce an hidroextraction.

The final volume of the each extract obtained was 30 mL and were stored at refrigerator in sterile bottles for further use.

3. Results and discussion

In experimental variant V1, 18 Petri dishes were tested. On average 7-8 colonies of *Aspergillus* and *Penicillium*/Petri dish were grown. Of the six extracts used in three proportions each, only two samples have been shown to have a visible macroscopic effect on the inhibition of fungi culture.

This is the 1: 2 or 1: 3 cloves extract with the culture medium. The inhibition is reduced in the fungal colonies, the area being no more than 0.5 mm around the filter paper disc imbedded with the cloves extract. We believe that the method applicable to bacteria is not relevant for antifungal testing. In the disks used, the concentration of the extracts is much lower than an effective dose for the inhibition of mold species (Table1).

Table 1.

The observed results on the inhibition of plant hydroextracts on fungal cultures tested by the antibiotic method

Experimental variants /Plant species	**V1 – 24 h**			**V1 – 48 h**		
	V1.1	V1.2	V1.3	V1.1	V1.2	V.1.3
Results	Ø of the inhibition zone mm			Ø of the inhibition zone mm		
Cinnamomum zeylanicum	0.5	0.5	0	0.5	0.5	0
Laurus nobilis	0	0	0	0	0	0
Eugenia caryophyllata	0.5	0.5	0	0.5	0.5	0
Carum carvi	0	0	0	0	0	0
Coriandrum sativum	0	0	0	0	0	0
Anethum graveolens	0	0	0	0	0	0

Table 2.

Inhibition of fungal cultures caused by the inclusion of the hydro-extracts in the culture medium

Experimental variants /Plant species	**V2**				**V3**				**V4**
	V2.1	V2.2	V2.3	V2.4	V3.1	V3.2	V3.3	V3.4	96h
Results	Percentage development of colonies, relative to the volume of culture medium				Percentage development of colonies, relative to the surface of the plate				the degree of colony development %
Cinnamomum zeylanicum	70	50	20	100	0	0	0	100	20
Laurus nobilis	75	30	50	100	70	50	30	100	15
Eugenia caryophyllata	60	40	10	100	0	0	0	100	5
Carum carvi	80	80	50	100	100	90	90	100	30
Coriandrum sativum	80	60	50	100	100	100	90	100	50
Anethum graveolens	90	70	50	100	100	100	100	100	30

Fig. 1. Inhibition of caraway, coriander and dill extracts at 96 hours after inoculation

By including plant extracts in both the liquid culture medium and the agarized medium, the fungicidal effect of all plant extracts is evident at all concentrations tested. The inhibition of colony development relative to the control and relative to the concentration used is so evident that it can be estimated in the percentage of colony development either in the volume of the liquid medium or on the surface of the agar medium. Each of the Petri plates was treated on the right half with plant extracts, observing an obvious inhibitory effect thereof.

The order of extracts used is the following: *Carum carvi*, *Coriandrum sativum*, *Anethum graveolens* (Figure 1).

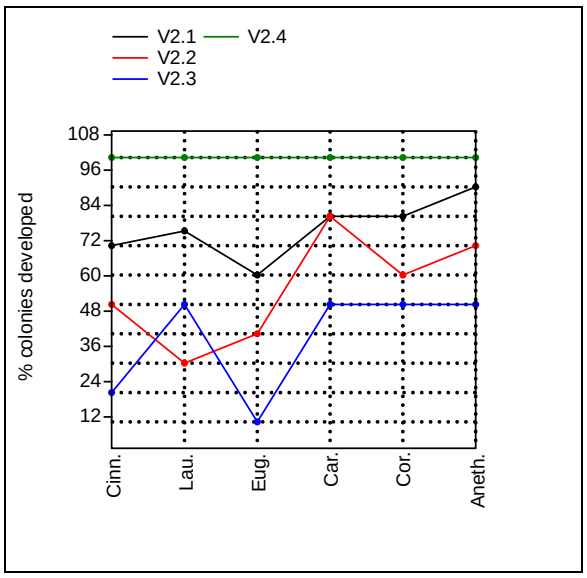

Fig. 2. Inhibition of plant extracts included in the liquid medium

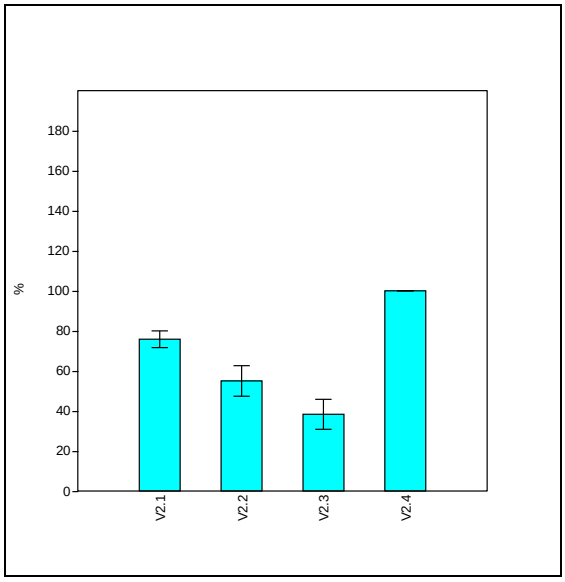

Fig. 3. Variation of the inhibitory effect, depending on the concentration of the extracts in the culture medium

The six species analyzed are grouped into two categories, both in terms of their climatic zones and the fungicide effect. On the one hand there are exotic spices from tropical and subtropical climates, on the other hand the species belonging to the *Apiaceae* family, coming from the spontaneous flora of the temperate zone (Figure 4, Figure 7).

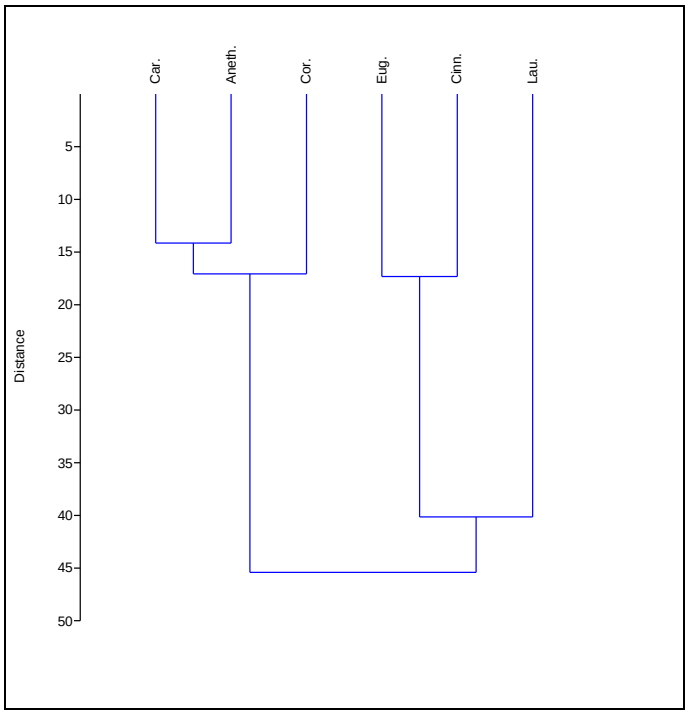

Fig. 4. Cluster analysis of the experimental variants for the six tested plant species

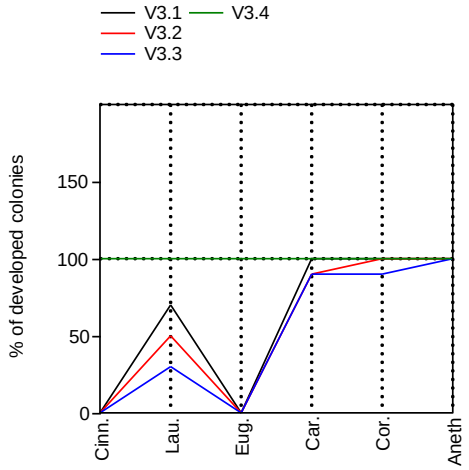

Fig. 5. Inhibition of plant extracts included in the solid medium

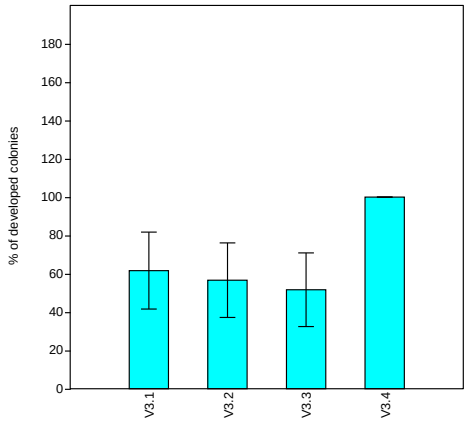

Fig. 6. Variation of the inhibitory effect, depending on the concentration of the extracts in the culture medium

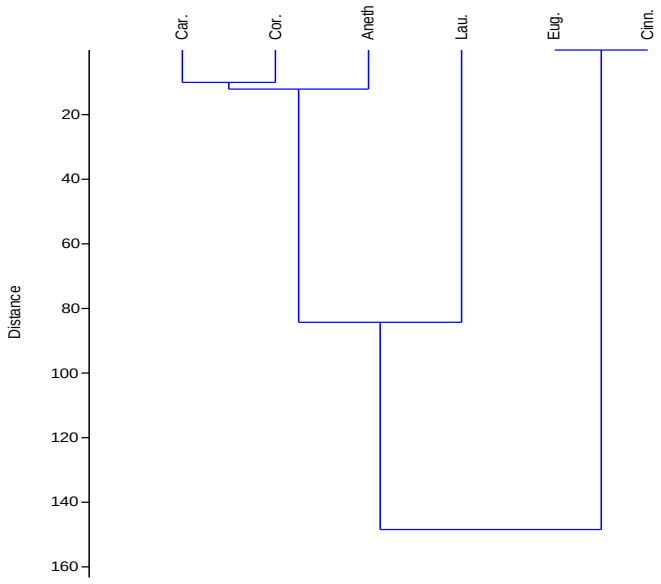

Fig. 7. Cluster analysis of the experimental variants for the six tested plant species

An analysis of the intensity of the inhibitory effect reveals that the most powerful effect is exotic spice extracts.

The cinnamon extract introduced into the liquid medium inhibits the development of colonies in varying percentages, depending on concentration, and introduced into the agar medium, completely prevents the growth of spores and colonies (Table 2, Figure 2, Figure 5). The previous studies reveal that also cinnamon oil in vapour phase is a strong inhibitor for fungal and bacterial growth [18, 23-26].

Laurel leaves have a pronounced inhibitory effect, although among the three exotic species, it is in third place (Figure 7). *Laurus nobilis* essential oils presented a good source of bioactive compounds such as 1,8-cineole, methyl eugenol, a-terpinyl acetate and linalool, which were considered as powerful bactericides [27-28]. *Laurus nobilis* etheroleum has antifungal activity probably due to the presence of the major monoterpenes and sesquiterpenes identified, likely acts interfering with the cell wall biosynthesis and ionic permeability of the membrane, and has deleterious effects on C. albicans biofilm adhesion and formation [29-31].

In the experiments carried out, the most potent inhibitor of mold species is the extract of cloves (Figure 2, Figure 5). In particular, the extract in all tested proportions included in the agar medium, completely inhibits spore development. Clove (*Syzigium aromaticum* L., family *Myrtaceae*) is considered to have an enormous potential as a food preservative against spoilage and pathogenic bacteria [11, 32]. The phytochemical constituents of this plant includes eugenol, transcaryophyllene, a-humulene, eugenol acetate, syzygin A, syzygin B, caffeic acid, ferulic acid and ellagic acid [2, 7, 33]. The extract of cloves used as such in various concentrations, as well as in nanocomposites, has certain therapeutic effects [34-35].

The second group is that of the *Apiaceae* family. This family is rich in phytochemicals and secondary metabolites which are potential source of drugs such as terpenoids, triterpenoid saponins, flavonoids, coumarins, polyacetylenes and steroids [36].

Carum carvi is known to be one of the species with potential for aflatoxigenic reduction *Aspergilli*, which are cosmopolitan fungi with air-borne conidia as infective propagules, they routinely contaminate foods, feeds and agricultural commodities such as peanuts, corn, pistachio nuts and oil seeds all over the world [37]. The results clearly show that the EOs of *Carum carvi* may have potential for use as natural preservatives in controlling aflatoxins contamination of foods, feeds and agricultural commodities in practice [38].

The antibacterial activity of *Carum carvi* could be attributed to the high polyphenolic compounds present in the extract [9, 31]. In studies conducted, 3:10 caraway extract with culture medium, inhibits the growth of mold colonies by 50%.

Although the bibliographic studies show coriander extract as effective against Candida, Fusarium, Aspergillus and Penicillium strains [5-6, 8, 39-40], in our experiments, umbellifer species inhibit the growth of fungal cultures in proportions of 10-50%. At the level of use in preserving food, the effect is beneficial and consistent with similar studies. Coriander essential oil could inhibit the growth of fungal in the cake and could be thus, used as a potential antifungal agent in foodstuffs especially those containing lipids [41-43]. The *Coriandrum sativum* etheroleum showed excellent antifungal activity against seed borne pathogens of paddy: *Pyricularia oryzae*, *Bipolaris oryzae*, *Alternaria alternata*, *Tricoconis padwickii*, *Drechslera tetramera*, *Drechslera halodes*, *Curvularia lunata*, *Fusarium moniliforme*, *Fusarium oxysprorum* [10, 20, 30, 44].

In the case of fungal strains grown in symbiotic relation with ant species, the coriander has shown an inhibitory capacity between 23, 3, and 100% of the fungal biomass [24, 45].

Anethum graveolens is a widely grown species with certain aromatic qualities.Various pharmacological actions of *A. graveolens* such as antimicrobial, antispasmodic, antidiabetic, anti-

hypercholesterolaemic, and anti-inflammatory have been reported [12, 46-47]. In relation to saprophytic fungi, it diminishes the ability of colony development in liquid nutrient medium and also has a retarding effect on colonies (Table 2).

4. Conclusion

All plant species, through their hydrodextracted compounds, demonstrate significant abilities in inhibiting the development of saprophytic molds. The effect is stronger on solid culture media, especially at the level of colonies developed on surfaces. The inhibitory effect is persistent over time, being visible even at 96 hours after contact with fungal colony (Table 2). Efficiency is better as the plant extract is more concentrated (Figure 3, Figure 6). The plant extracts tested can be used to impregnate solid food packaging, being able to prevent or slow the growth of mold colonies.

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