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Optimization of the extraction parameters of *Aloe Vera* polyphenols and study of antioxidant and antifungal activities: application to molds isolated from durum wheat

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Abstract: *The main objectives of this work are to optimize the extraction parameters, to test the antioxidant activity of Aloe Vera extract and to study the impact of this extract on deteriorating molds of Algerian variety of wheat (CIRTA). The extraction was optimized by central composite design. Determination of the polyphenols, flavonoids, and proanthocyanidins was performed by using colorimetric assays. Identification and quantification of phenolic compounds were performed by RP-HPLC-UV method. The antioxidant activity was tested by three methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and CUPRAC (Cupric reducing antioxidant capacity), the antifungal activity of Aloe Vera extract on isolated strains from durum wheat were tested by dilution in a solid medium method. The optimum of total phenolic got was1,044 x 104 µg GAE/g of dry extract. The extract is rich in polyphenols, flavonoids, and proanthocyanidins. The analysis of phenolic compounds of Aloe Vera by RP-HPLC-UV revealed seven phenolic compounds. Strong antioxidant activity was obtained for Aloe Vera extract. Purification and microscopic study of isolated strains gave the possibility of identifying four strains: Alternaria spp1, Alternaria spp2, Penicillium spp, and Aspergillus spp. the antifungal potential of Aloe Veravaries according to the fungal genera and the concentrations of extract used.*

Keywords: Optimization, Polyphenols, Antioxidant activity, Antifungal activity, *Aloe Vera,* Mold, wheat

Introduction

The cereals sector makes up one of the important bases of food industry in Algeria. This importance results of the dominating place which occupy the cereals and their derivatives in the human consumption, in particular, the semolina (couscous and pastes) and the flour (bread), as in the animal feeds (wheat bran, wheat straw) [1]. Wheat grains are rapidly altered if they are stored in unfavorable conditions [2]. Several phenomena are the cause (insects, micro-organisms, oxidation, etc. [3,4]. With the revolution in the Agro-food sector, it became necessary to maximize the production to ensure adequate food for the world's population. To do so, it is necessary to protect these products from any alterations. Among the micro-organisms of deterioration, the molds represent the group more diversified and the richest of several species. The molds can cause important losses by reducing quality and/or the quantity of stored wheat. On the enormous cultivated or collected quantities, an important part is destroyed and deteriorated each year during storage [5,6]. The microbial wheat contamination is the principal damage which will involve many problems. There is thus a reduction, quantitative and qualitative of the food value and a fall of the output of harvests [7]. The metabolites produced by mushrooms at the time of their growth are major elements in

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the deterioration of the foodstuffs like the changes of the taste of the food product for example [8,9,10]. They also emerge into serious medical problems, such as the risks of intoxication by mycotoxins [11,12]. Because of its effectiveness and its easy and practical application, the use of the chemical products makes up for the present time the technique most used to fight against the harmful molds [12]. However, the intensive and ill-considered use of these products caused contamination of the biosphere and food chain, an eradication of auxiliary fauna and the appearance of the resistant micro-organisms. These dangers led WHO to prohibit the use of certain chemical fungicides [13]. It became essential the search for new molecules by considering other criteria that the effectiveness. We directed this research towards the biological fight by the use of antioxidant and antifungal natural substances being able to constitute an alternative solution with the chemical products. The extracts of the plants appear among these natural substances. The aromatic plants make up a very important natural wealth whose valorization requires a perfect knowledge of the properties to emphasize. The properties of the plants depend on the presence of varied bioactive agents and pertaining to various chemical classes [14]. Algeria, by its geographical location, offers a rich and various vegetation. Numerous aromatic plants push there spontaneously. The interest carried to these plants did not cease growing during these last years. For this purpose, Aloe Vera has interested us in for its considerable medicinal properties [15,16]. Many biological properties associated with the species of Aloe come from the internal leaf gel. Most research has been centralized on the biological activities of different aloe species, including antibacterial and antimicrobial activities of non-volatile leaf gel constituents [15,17,18,19], but the effect of its extracts on molds that develop during the storage of wheat has not been studied. Availability, lack of toxicity, and richness in bioactive compounds allow the use of Aloe Vera as an interesting alternative of chemical fungicides [15,16, 20]. In this context, the aim of this work is to highlight antioxidant and antifungal activity of Aloe's extracts and to study their action on the growth of durum wheat degradation.

Material and methods

Plant material

We harvested manually *Aloe Vera* plants from the Food and Agri-Food Institute (INATAA), University of Constantine**.**

Optimization of extraction of phenolic compounds from *Aloe Vera*

Aloe Vera polyphenols are extracted by maceration and ultrasound [21]. In this work, we used a central composite design CCD to determine the effect of maceration time (X 1) and time of ultrasound (X 2) on the total polyphenols content of *Aloe Vera*(Y) extracts. The experiment design allowed optimization of extraction with the best total polyphenol rate. The model studied is a two-factor model. This experiment design requires eight experiments representing combinations of three levels assigned to each of the two factors while taking the corresponding response. We have added five central points (00) to support this experience plan; which gives 13 tests. *Aloe Vera's* plant was dried, cut into small pieces, extracted to methanol in a beaker and then placed in an ultrasound. Then the extracts were filtered, the plants were re-extracted three times, and the combined extracts were used for experiments. The solvent was evaporated by a rotary evaporator. The mathematical model postulated is a second-degree model with order interactions 2. It reflects the dependence of the response studied Yen function of the two coded variables (X 1 and X 2).

$$\mathbf{Y = b_0 + b_1X_1 + b_2X_2 + \ b_{11}X_1X_1 + b_{22}X_2X_2 + b_{12}X_1X_2}$$

With: Y: response studied;

X 1, X 2: coded forms of real variables;

b 0: constant which expresses the overall average effect;

b 1, b 2: linear coefficients (the main effect of each variable);

b 11, b 22: quadratic coefficients;

b 12: interaction coefficient (interaction between the two variables).

For each coded variable (Xi) five levels, -α, -1, 0 and 1, +α are assigned. -α and+α represent extreme values (min, max). For orthogonal and rotary design, the coded value of α was determined by

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STATISTICA 7.0 (StatSoft, Inc., Tulsa, OK, USA) at 1.414. We base the selection of levels of maceration time and ultrasound time on preliminary tests and references to the bibliography

Content of total polyphenols

The dosage of total polyphenols was performed by the Folin Ciocalteu reagent method [22].

Determination of Flavonoids

The flavonoids assay was performed according to the method of aluminum trichloride ($AlCl_3$)[23].

Determination of proanthocyanidins

The amount of tannins was determined by the method described by Skerget et al. [24].

Analysis RP-HPLC-UV

We carried the quantitative and qualitative analysis of phenolic compounds out by HPLC Shimadzu model LC -20 AT equipped with four pumps and a UV/Vis Shimadzu SPD -20 AV detector. We performed automatic chromatographic separation on an analytical column C 18 inverted waves PR PR (150 mm x 4, 6 mm, 5 um). The column temperature was set at 40 ° C. The elution gradient comprises the mobile phase A (water, ammonium formate 5 mM and formic acid at 0.1%) and the moving phase B (methanol, ammonium formate 5 mM and formic acid) The solvent flow was maintained at 0.5 ml/min and the injection volume was fixed at 4 µ l. In this study, fifteen phenolic compounds widespread in plant material were qualified and quantified (Gallic acid, chlorogenic acid, Protocatechuic acid OH, p- benzoic acid, Catechin, Vanillic acid, Caffeic acid, Syringic acid, Epicatechin, p-Coumaric acid, Ferulic acid, Quercetin, Rutin, Apigenin, luteolin). We obtained phenolic standards from Sigma-Aldrich (Munich, Germany). The detection limit (LOD) and the limit of quantification (LQ) of the method described in this study depended on the calibration curve based on six measurements.

Antioxidant activity

Evaluation of the antioxidant activity is carried 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and cupric reducing antioxidant capacity (CUPRAC) methods.

DPPH Method: The evaluation of the antioxidant activity was done by the method of Blois [25].
ABTS Method: The evaluation of the antioxidant activity was carried out by the ABTS free radical scavenging method described by Re et al. [26].

CUPRAC Method: We realized the evaluation of the antioxidant activity by the method of Özyürek et al. [27]. The IC50 index of extract and reference antioxidant Butylated hydroxyanisole (BHA) has been determined.

Isolation of mold and determination of antifungal activity
Sampling

Durum wheat samples CIRTA variety were brought from Constantine's Cereal and legumes Co-op, Algeria (CCLS), these grains are not previously processed by chemical fungicides. We randomly selected five seeds; we put them in Petri dishes containing the Sabouraud medium (seed in the center and one in each quarter of the circle) and we incubate them at 28 ° C for seven days. We made three replicas per sample. The identification of different species of fungi was based on fungal morphology according to Pitt and Hocking [28].

Evaluation of the antifungal activity of Aloe Vera's extract

The antifungal activity of extract on strains isolated from durum wheat was assessed using method of Gakuubi et al. [29] and Kordaliet al. [30].

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Statistical analysis

The means plus or minus the standard deviation of the three replicates of the content of total polyphenol, flavonoid and proanthocyanidins; percentages of antioxidant activity andantifungal activity; and graphic representations, were performed with Excel 2013 (Microsoft Excel Version 3. 2013, Microsoft Corp., Redmond, WA, USA). Means were compared by a single factor analysis of variance (ANOVA) analysis followed by a post-hoc Tukey test using STATISTICA 7.0 software (StatSoft, Inc., Tulsa, OK, USA). The superscript letters a, b, c, d, and e show a significant difference at the 0.05 significance level. We generate the test matrices with Minitab 17 software (Minitab Inc., State College, PA, USA). Surface plots and the optimization of the factors were made by STATISTICA 07.0 software (StatSoft, Inc., Tulsa, OK, USA). We realized correlation test between the total polyphenol content and the antioxidant activity and the analysis of the principal components (APC) out by the XLSTAT 2019 software (Addinsoft, 2019.XLSTAT statistical data analysis solution, Paris, France).

Results and discussion

Total polyphenol content

We got a satisfactory answer compared to the change of the data of the quadratic model of regression; the coefficient of determination for this model is of 98.73%. The mathematical model got is very significant (p<0, 0001). The time of maceration, the squares of time of maceration and the time of ultra-sonication have significant effects on the answer (p< 0.05). The linear term of the time of ultra-sonication (B) and the interaction between factors (AB) is not significant. We express the predicted polynomial model in terms of the codes factors as follows

Total polyphenol =-23 952 + 16.24 A + B 1334 - 0.011032 A * A - 14.37 B * B - 0.0866 A * B (3)

Based on these results, the optimal values of the total polyphenol levels are in the red zone limited by the points: maceration time (400-750min) and ultra-sonication time (40-50min) (Figure 1).The optimum factors have been calculated at a 0.96 desirability(Figure 2).

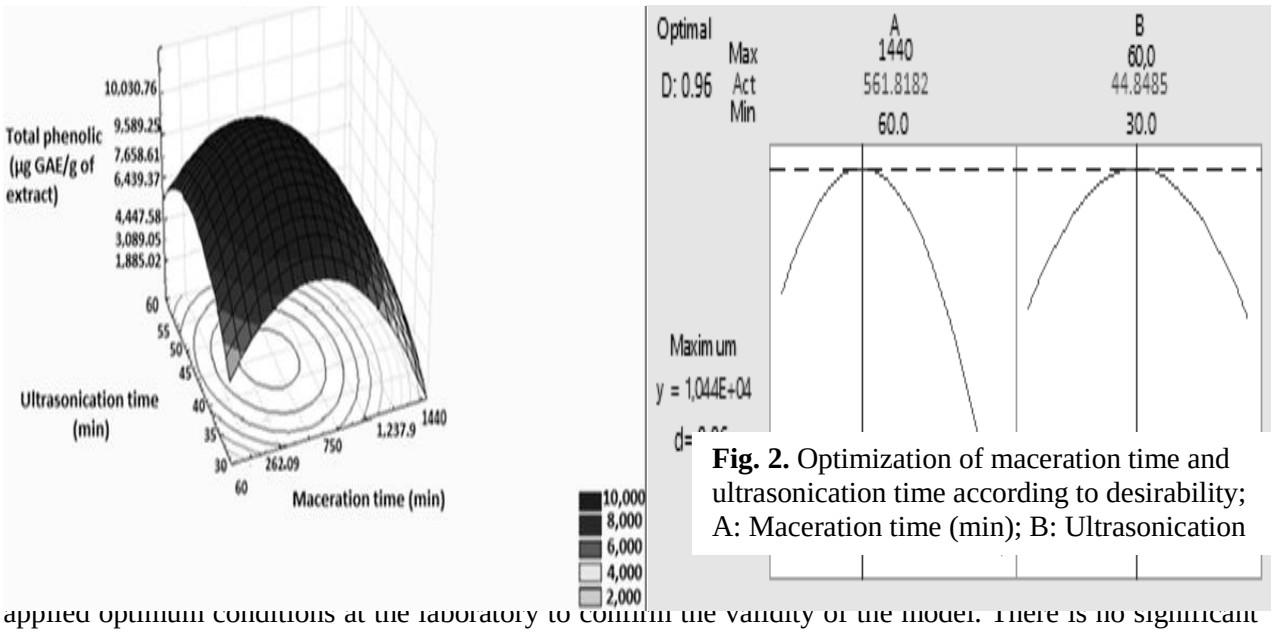

Fig. 2. Optimization of maceration time and ultrasonication time according to desirability; A: Maceration time (min); B: Ultrasonication

applied optimum conditions at the laboratory to confirm the validity of the model. There is no significant difference between the measured and predicted values, which confirms the validation of the model. In

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other works, carried out in this context, Nejatzadeh-Barandozi [31] studying the *Aloe Vera* leaves from IRAN, they found that the concentration of 78.2 ± 4.03mg Eq AG / 100g dry weight. Kumar et al. [32] analyzed the effect of climate change on the total phenolic content (TPC) of *Aloe Vera* collected from different climatic zones of India. The values range from 32.9 to 65.7 mg EGA per g dry weight. Vastrad et al. [33] revealed that *Aloe Vera* leaves revealed total polyphenol contents according to the solvent used for the extraction, the values got are (138.13 mg / g) with extraction with ethanol followed by extraction with methanol (95.20 mg / g) and (94.42 mg / g) with extraction with water. This difference could be attributed to climatic conditions, harvest time and cultivars, method and solvent extraction [34].

Total flavonoid content

The concentration of flavonoids got is equal to 2.45 ± 0.62 mg Eq Q / g of dry extract. Our results are inferior to those found by Muthukumaran et al. [35] which showed that the concentration of flavonoids varies between 0.05 - 0.01 mg quercetin / g. Nejatzadeh-Barandozi [31] got a flavonoid content in the leaf equal to 5.3 ± 0.38 mg Eq Q / 100g of the dry weight of *Aloe Vera* collected in IRAN regions.

Proanthocyanidin content

The concentration of proanthocyanidins of *Aloe vera* is equal to 5.925 ± 0.09 mg Eq of tannic acid / g of extract. Amoo et al. [36] found concentrations ranging from 0.04 ± 0.039 to 2.90 ± 0.447% of the dry matter of A. arborescens.

RP-HPLC-UV analysis

We have identified seven phenolic compounds in Aloe vera extract and which are: Protocatechuic acid, p-OH benzoic acid, Caffeic acid, p-Coumaric acid, Quercetin, Luteolin, Chrysin (Table 1).

Table1.RP-HPLC-UV analysis

	compounds	**Retention time (min)**	**% RSD (Area)**	**R2**	**LOD**	**LOQ**	**Quantization (analyte µg / g of extract)**
1	Gallic acid	10.841	0.220	0.999	0.021	0.066	ND
2	chlorogenic acid,	15.910	0.851	0.999	0.041	0.129	ND
3	Protocatechuic acid	21.055	0361	0.998	0.035	0.110	1058.851
4	OH p-benzoic acid	23.279	0.493	0.997	0.041	0.122	3548.585
5	catechin	25.066	0.838	1.000	0.023	0.078	ND
6	Vanilic acid	26.039	0.169	0.998	0.061	0.189	ND
7	caffeic acid	28.008	0.551	1.000	0.001	0.026	100251
8	epicatechin	29.009	0.439	0.999	0.029	0.095	ND
9	p-Coumaric acid	30.562	0.202	0.999	0.013	0.035	4573.251
10	Ferulic acid	33.258	0.212	0.999	0.015	0.039	ND
11	quercetin	35.002	0.224	1.000	0.041	0.125	2541.231
12	apigenin	39.000	0.252	1.000	0.015	0.048	ND
13	luteolin	41.833	0.256	0.989	0.043	0.150	1548.258
14	rhamnetin	45.041	0.245	0.999	0.051	0.158	ND
15	Chrysin	52920	0.358	0.984	0.025	0.124	118.25

These results are different to those obtained in several studies: According to López et al. [37], The results showed that catechin, sinapic acid, gentisic acid and epicatechin, Kaempferol, Apigenin, gallic acid were the most abundant compounds in the extract of leaves of *Aloe vera*, while genic acid, epicatechin,

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and quercetin were the most abundant compounds in the extract of Aloe Vera's gel. In addition, Bhalla and Chauhan [38] also identified three compounds: quercitrin, kaempferol and apigenin in Aloe vera leaf extract that may have antioxidant activity. Several flavonoids have been detected and quantified in the genus *Aloe*, the major flavonoids of this plant are naringenin, apigenin, isovitexin, and dihydro-isorhamnetin [39]. The chemistry of the Aloe plant has been studied, but the focus has been on the gel and its well-known therapeutic properties. However, various phenolic compounds such as chlorogenic, caffeic, p-coumaric and ferulic acids and aloin derivates have been detected [37, 40, 41,42].

Antioxidant activity

We have determined the IC50 of the extract and BHA, the results are summarized in Table 2:

Table 2. Half-maximal inhibitory concentration IC50 values.

	DPPH	ABTS	CUPRAC
Aloe vera	2.359 ± 0.26^a	2.216 ± 0.25^a	3.051 ± 0.05^a
BHA	2.299 ± 0.56^a	2.235 ± 0.85^a	2.991 ± 0.25^a

Values are means clustering ± SD, n = 3, the superscript letter a show that there is not a significant difference at the 0.05 significance level. DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); CUPRAC: cupric Reducing antioxidant capacity; BHA: Butylated hydroxyanisol

No significant difference (p> 0.05) was got between the IC50s of the *Aloe Vera* extract and BHA. *Aloe Vera* extract shows an important reducing power. This activity may be attributed to certain enzymes such as Glutathione peroxidase, superoxide dismutase and the richness of *Aloe Vera* extract in phenolic compounds [32,37, 43]. We performed a correlation test to verify the correlation between polyphenol rate variation and the antioxidant activity of *Aloe Vera* extract. The values of the Pearson coefficients and the values of p are recorded in Table3.

Table 3. Correlation Between Antioxidant Activity and Total Polyphenol Content of *Aloe Vera Extract*

Antioxidant activity	**DPPH**		**ABTS**		**CUPRAC**	
Aloe Vera polyphenols	r	P	r	p	r	p
	0,996	0,000	0,974	0,006	0,996	0,000

r: Pearson coefficient, P: probability

We observed a strong positive correlation between the total polyphenol content and the antioxidant activity tested by the three methods (DPPH, ABTS, and CUPRAC). Several studies have shown that *Aloe vera* leaf has a significant antioxidant activity due to its richness in phenolic compounds and flavonoids. These compounds are located in the outer skin of the leaves and are abundant in the *Aloe vera s*pecies [44]. Our results confirm those found by several researchers [45, 46, 47].

Mold Isolation

We found that the durum wheat sample is contaminated with four kinds of fungi: *Penicillium, Alternaria spp1, Alternaria spp2, Aspergillus.* According to the literature, the most common species in wheat are *Penicillium and alternaria*[48, 49, 50,51, 52]. *Penicillium* can develop at Aw around 0.7 to 25 ° C, and therefore grow in water-poor foods such as wheat during storage [53]. Botton et al. [54] pointed out that, during storage, osmophilic molds can develop, which causes substrate acidification; especially *Aspergillus* and *Penicillium, Alternaria* are hygrophilous genera, their existence is due to the increase of the grain moisture at the time of harvest. If the storage conditions are unfavorable, these molds are factors of biological deterioration of the wheat affecting the quality of the raw materials, or the sanitary quality by the secretion of mycotoxins [55]. In other studies, Joshaghani et al. [56] isolated seven strains from stored

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wheat: *Alternaria, Aspergillus, Fusarium, Aspergillus, Cladosporium, Penicillium* and *Rhizopus.* Ennouari et al. [57] showed that the most abundant fungal strains in wheat are: *Alternaria spp., Fusarium spp.* and *Aspergillus spp*. (14%). Anžlovar et al. [58] isolated five strains from wheat that are *Alternaria alternata, Alternaria infectoria, Aspergillus flavus, Epicoccum nigrum, and Fusarium poae.*

Antifungal activity

Figure3 summarizes the effect of different concentrations of *Aloe Vera*extract onthe four strains of durum wheat variety CIRTA

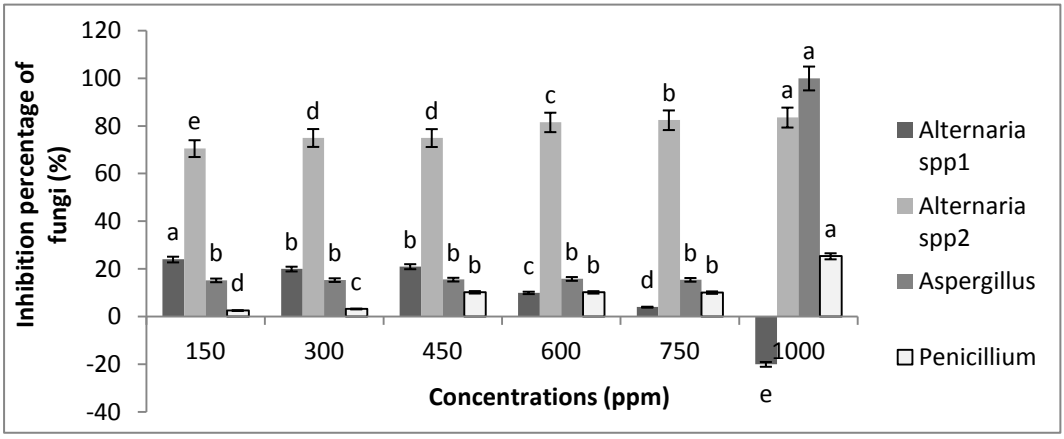

Figure 3. Inhibition rate of isolated strains by *Aloe Vera* extract. The superscript letters a, b, c, d, and e show a significant difference between antifungal activities at different concentrations of extract (ppm) at the 0.05 significance level

For the *Alternaria spp1* strain; the extract showed a moderate activity which regresses with the augmentation of the concentration, but at the concentration of 1000 ppm, the negative effect appears (the increase of the growth of the mold compared to the control). All concentrations of *Aloe Vera* extract solutions have strong activity against *Alternaria spp2* strain. While *Aloe Vera* extracts showed low antifungal activity on the *Aspergillus* strains at Petri dishes that contain concentrations of 150-750 ppm. However, complete inhibition of the growth of this mold (100%) was obtained in the petri dish containing 1000 ppm of *Aloe Vera* extract. While low activity was recorded on the *Penicillium* strain.The effect of extract varies with the fungal genera and species and the used concentrations. Inhibition of growth of *Alternaria spp2* and *Aspergillus* at 1000 ppm is because of the inhibition of conidial germination. [59,60] and also inhibits mold growth [15,61]. Several compounds of *Aloe Vera* can contribute to this antifungal activity, including phenolic compounds. To verify the correlation between the variation of concentration of polyphenol and antifungal activity, we performed a Principal Component Analyzes (PCA) for testing the correlation between antifungal activity (expressed in%) and the different concentrations of the polyphenols (figure4)

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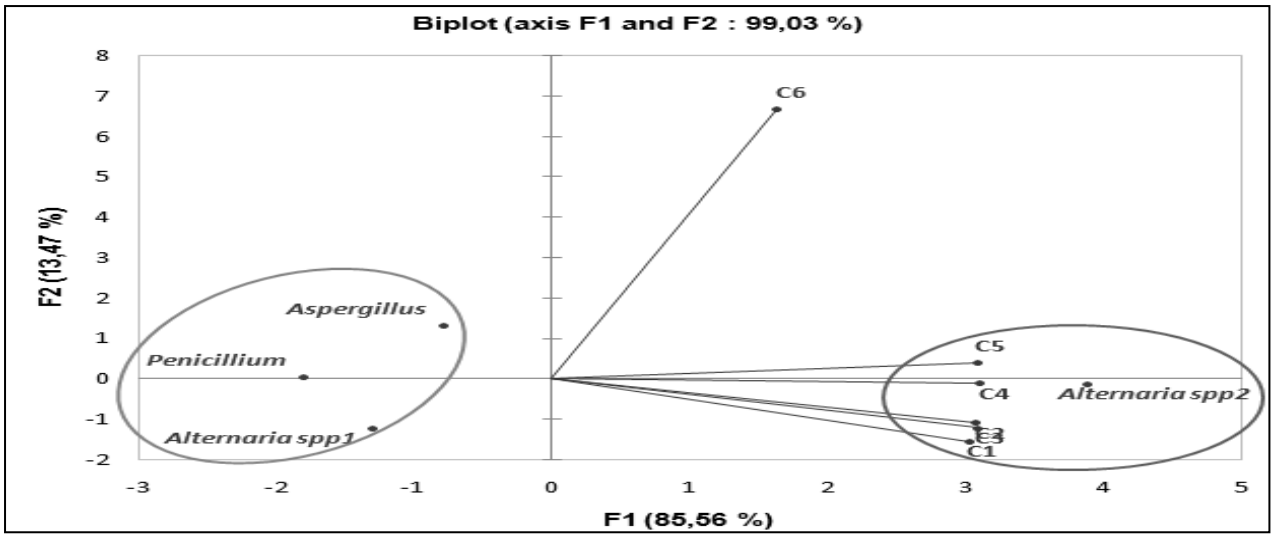

Fig. 4. Principal Component Analyzes (PCA) performed on antifungal activity (expressedin%) on isolated strainsaccording to the different concentrations of the polyphenols. (C1: 150ppm, C2:300ppm, C3:450ppm, C4:600ppm, C5:750ppm, C6:1000ppm.

The PCA biplot is shown in the F1-F2 plane (Figure 4). Axis 1 represents 85.56% and Axis 2 represents 13.47% of the total variability. Axis 1 is positively explained by the concentrations C1, C2, C3, C4, and C5. A strong correlation was observed between C2 and C3. Axis 2 is positively explained by C6. On the map representing the individuals, discrimination is observed according to the genus of the molds, in particular for *Alternaria* spp2.This antifungal activity is attributed to the variation in the concentration of phenolic compounds of *Aloe Vera* compounds that are considered active antimicrobial agents [61,62]. This antifungal can also be related to flavonoids from *Aloe Vera* extract [63,64] and some Anthraquinones such as aloin and aloe-emodin [65]. The antifungal activity on *Penicillium,Alternaria* spp1 and *Aspergillus* is not related to the change in polyphenol concentration.This difference in sensitivity to phenolic compounds between different species belonging to the same genera and between the various fungal structures of the same genus: spores, sclerotitis, and mycelial fragments. Knowing this activity is not general for many isolated durum wheat mold, some of them may even consume the extracts as a source of carbon, degrade them or transform them, which may explain the inefficiency of some of these molecules on certain microorganisms and this may explain the increase in the strain's growth *Alternaria* spp1 at a concentration of 1000 ppm of extract. The more or less advanced biodegradation of this molecule may explain the weak antifungal activity of the extract on *Penicillium* and *Aspergillus* (for the concentrations: 150-800ppm) [66]. One factor affecting the intensity of the antifungal action is the applied dose of the extract [67].

Conclusion

The results show that the extract of *Aloe Vera*is rich in polyphenols, flavonoids, and proanthocyanidins. The RP-HPLC-UV analysis of the phenolic compounds of *Aloe Vera* reveals seven major compounds which are: Protocatechuic acid, p-OH benzoic acid, Caffeic acid, p-Coumaric acid, quercetin, Luteolin, Chrysin. According to our study, the extracts of *Aloe Vera* showed a very strong antioxidant activity close to BHA. For mycological analysis, we isolated and purified four strains of mold from samples of durum wheat variety (CIRTA) not treated with fungicides and which are*: Alternaria spp1, Alternaria spp2, Aspergillus* and*Penicillium).* The extract did not show the same antifungal power on isolated strains. The antifungal activity depends on the applied dose of the extract, the genus and the species of mold tested.

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