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Chemical and Phytochemical composition of Moringa seed powder and Antifungal activity of seed extracts against seed borne fungi[1]

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Abstract: The aim of the current study were to determine the quality of *Moringa oleifera* seeds and to detect the *in-vitro* antifungal activity of seed extracts and their phytochemical and elemental composition. Seeds of moringa recently collected or stored for one to three years were contaminated with fungi with an average of 48.44% on PDA medium. The percentage of fungal infection was higher in seeds produced during season of 2011(stored for 3 years), followed by seeds produced at season of 2012 with significant difference (87.50 and 50.00% respectively). Moringa seeds recently collected during season of 2014 showed low fungal densities than the corresponding figures of stored seeds for 1-3 years. Fourteen species which belong to nine genera were detected and they were classified as, *Alternaria alternata* (11.82%), *Aspergillus candidus* (4.32%), *A. flavus* (6.34%), *A. niger* (14.46%), *A. regulosus* (1.87%), *A. sydowi* (3.08%), *A. terreus* (6.09%), *Chaetomium globosum* (7.55%), *Fusarium solani* (2.35%), *Helminthosporium* sp. (5.97%), *Macrophomina phaseolina* (6.88), *Nigrospora sphaericica* (16.69%), *Rhizoctonia solani* (4.19%) and *Rhizopus nigricans* (8.35). The ethanolic seed extract was found to be more effective than the water extract. The ethanolic seed extract at 100% cause increase in zone of inhibition in all tested fungi reaching 2.70-3.77mm, whereas, at 50% concentration reaching between 1.40-1.93mm. The aqueous extract of moringa seeds at 100% concentration showed moderate activity against all tested fungi (inhibition zone between 0.43-1.33 mm) compared with the standard fungicide (inhibition zone 4.23 - 4.90 mm).
The results of the phytochemical analysis revealed differences in the presence of components among the seed extracts. The total phenol was decreased from 7.00 to 3.19 mg/g (54.4% decrease) and total flavonoids from 0.697 to 0.550 mg/g (21.09 decrease) in moringa recently collected (2014) and stored seeds for 3 years respectively. The percentage of nitrogen, phosphorus and potassium were high in recently collected seeds than in the stored seeds, as the percentages were 5.6, 0.64 and 1.18 % in recently collected seeds respectively and reduced to 5.1, 0.91 and 1.11 % respectively in old seeds for 3 years. GC/MS analysis of recent collected moringa and stored seed extracts led to the identification of different compounds. Extract of recent collected seeds during season 2014 contained some compounds varied in their chemical composition and molecular weight than old seeds of 3 years.
Keywords: *Moringa oleifera,* antifungal activity, GC/MS analysis, seed born fungi, seed extracts

1. Introduction:

Moringa oleifera (Linn) is a highly valued plant distributed in many countries. It has an impressive range of medicinal used with high nutritional value. Different parts of this plant contain a profile of important minerals and are a good source of protein, vitamins, carotene, amino acids and various phenolics[1].

Many reports described. *M. oleifera* as highly potent anti-inflammation[2], hepatoprotective[3], anti-hypertensive[4] and anti-tumor[5]. Previous studies have reported that various parts of *Moringa* root, bark and stem including seeds possess antimicrobial properties[6-9]. Also, its seed has strong coagulative and anti-microbial properties against plant pathogenic fungi[9-12] and against some food borne microorganisms tested by paper disc method[13].

Since fungicides are expensive and cause serious environmental pollution , control strategies are today directed towards replacing the use of hazardous chemical fungicides by environmentally natural products[9-14]. The fungicidal effect of moringa extracts on some soil-borne fungi such as *Rhizoctonia*, *Pythium* and *Fusarium* spp. was recorded by many investigators[15]. Moreover, Dwivedi, S.K. et al[16]report that *Moringa oleifera* extracts (75 % v/v) showed significant inhibition in the mycelial growth of *Fusarium solani. M. oleifera* provides a rich and rare combination of zeatin, quercetin, b-sitsterol, caffeoylquinic acid and kaempferol which have antifungal and antibacterial activities[17]. Other investigators Adandonon, T.A. et al [18], tested the aqueous extract of moringa seed as a bio fungicide to replace the synthetic ones which are currently being used to devise an organic approach. While, Jamil, A et al [19] reported that *M. oleifera* extract showed antifungal activity against *Mucar mucedo* and *Aspergillus niger* more strongly than *Aspergillus tamari* and *Rhizoctonia solani*. Beside, Mohammed, A et al [20]studied the antifungal activity of *M. oleifera* extract against *Alternaria*, *Colletotrichum*, *Curvularia* and *Fusarium* spp. pathogenic fungi.

The present study has therefore been undertaken to detect, the seed-borne fungi associated with *Moringa oleifera* seeds collected during seasons of 2011-2014 and to detect the antifungal activity of the ethanolic and water extracts of *Moringa oleifera* seeds against some important phytopathogenic fungi and also to determine the phytochemical and elemental composition of the seed powder.

2. Materials and Methods:

2.1 Moringa seeds:

The source of *Moringa oleifera* pod coats (seeds) collected during seasons of 2011-2014 were kindly obtained from Egyptian Scientific Society of Moringa (ESSM), National Research Centre, Cairo ,Egypt.

2.2 Isolation of moringa seed borne fungi:

Laboratory analysis was done to detect the seed borne fungi associated with moringa before sowing according to[21]. One hundred seeds were surface disinfected by soaking in 2% sodium hypochlorite for 3 min, followed by 70% ethanol for 2 min and then thoroughly washed in sterile water. Drain excess water, dried between two layer of sterilized filter papers. The seeds were platted on potato dextrose agar (PDA) medium at rate of 4 seeds/dish. The plates were incubated at 27±2˚C for 7 days. Fungi growing from the seeds were isolated, purified and identified according to Barnett, H.L et al, Domsch, K.H et al, Samson, R et al [22-24].

2.3 Preparation of moringa seed extracts:

2.3.a Aqueous extract:

Seeds of moringa plants were collected in clean polyethylene bags, washed with distilled water, dried in shade and then grounded into powder. Ten g of the ground seeds were measured into a conical flask and 40ml of sterile distilled water was added and left properly on the shaker at 100 rpm for 24 hrs after which the extract was filtered and squeezed through three layer of muslin cloth. The filtrate was then centrifuged at 2000 rpm for 5 minutes after which it was decanted and the supernatant was sterilized by using the membrane filtration unit. The filtrate obtained was stored in sterile bottles and kept at 4˚C for antifungal activity[25].

2.3.b Ethanolic extract:

A 10g of powdered seed materials were soaked in 40ml of 95% ethanol for 5 days at room temperature and filtered as mentioned before. The filtrate was dried using a rotary evaporator at 60˚C[26].

2.4 Antifungal activity assay:

Antifungal activity of aqueous and ethanolic extracts of *M. oleifera* seeds against six pathogenic fungi associated with moringa seeds using agar well diffusion method with sterile cork borer of size 6.0 mm according to Bobbarala V. et al [27]. Cultures of 72 hours old grown on PDA medium were used for inoculation of fungal species on PDA plates. An aliquot (0.2ml) of inoculums was introduced to molten PDA and poured in to a petri-dish. After solidification, the appropriate wells were made on agar plate and 500 µl of seed extracts were filled in the well. Incubation period of 4-5 days at 27±2˚C was maintained for observation of antifungal activity by measuring the inhibition zone of fungal growth surrounding the well. The zone of inhibition was measured in mm and the experiment was carried out in triplicates. Synthetic fungicide (8 hydroxy quinolin, 100µg/ml) was used as positive control.

2.5 Chemical composition of seed powder:

Seed samples were ground in stainless steel mill with 0.5 mm sieve and kept in plastic containers for chemical analysis to determine the following traits:

2.5.1 Total nitrogen percentage: was determined using Micro-Kjeldahl method as described by Peter, L.P et al[28].

2.5.2 Macro (K, P and N) and micro (Cu, Mn, Zn and Fe) nutrients were extracted and determined as described by Chapman, H.D. et a[29], using atomic absorption spectrophotometer apparatus (Zeiss PMQ3). Phosphorus was measured in the digested solution using vanado-molybdate color reaction, according to the method described by Jackson, M.I. [30].

2.5.3 Phytochemical analysis

The seed extracts were subjected to phytochemical tests for :Total phenols (mg/g) according to Danial. H.D. et al[31], Total flavonoids (mg/g) by spectrophotometer according to Chang, M.Y. et al[32] and protein content was obtained by multiplying the nitrogen content by Nx6.25 according to method described by A.O.A.C. (1999)[33].

4. GC/MS analysis:

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1mL/min. The injector and MS transfer line temperature was set at 280˚C. The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

3. Results and Discussion

3.1 Percentage of the natural fungal infection:

Fig. (1): Moringa seeds infected with phytopathogenic fungi

It is clear from the data presented in Table (1) and Fig. (1) that seeds of moringa recently collected or stored for one to three years were contaminated with fungi with an average of 48.44% on PDA medium. The percentage of fungal infection was higher in seeds produced during season of 2011(stored for 3 years), followed

by seeds produced at season of 2012 with significant difference (87.50 and 50.00% respectively). On the other hand, recent collected seeds showed lowest densities of fungal contamination (18.75%). This result was confirmed by[9] who recorded that moringa seeds can infected with seed borne fungi.

Table 1. Percentage of fungal infection and fungal count (cfu/100 seeds) of moringa seeds recently collected (2014) or produced during seasons of 2011, 2012 and 2013 on PDA agar medium.

Seeds produced during season of	Infection %	Count of CFU/100 seeds
2011	87.50 A	293.8 A
2012	50.00 B	206.3 A
2013	37.50 BC	181.3 A
2014	18.75 C	56.25 B
Average	48.44	184.41

- Tests were rune in triplicates.
- Counts represent the number of fungi / 100 seeds incubated at 28±2˚C

Concerning the total fungal count as cfu, it is clear that the number of fungi per 100 seeds was ranged from 56.25 to 293.8 cfu/100 seeds on PDA with an average of 184.41. Moreover, moringa seeds recently collected during season of 2014 showed low fungal densities than the corresponding figures of stored seeds for 1-3 years. As, the counts were significantly increased from 56.25 to 181.3 cfu/seeds (3.22 folds increase) in recently collected and stored seed for one year respectively. These results can explained by the finding of Worang, R.L.[34] who reported that at the beginning of storage, most of the fungi that infected seeds were classified as field fungi, while after three months of storage, the existence of field fungi was generally replaced by storage fungi, or may be due to the antifungal activity of moringa seed content on fungal growth.

3.2 Frequency occurrence of fungi infected moringa seeds

Data in Table (2) showed that a great variation in types and in numbers of propagations among samples was noted and many fungal isolates which found in seeds of recently collected were absent in stored seeds. Fourteen species which belong to nine genera were detected and they were classified as, *Alternaria alternata* (11.82%), *Aspergillus candidus* (4.32%), *A. flavus* (6.34%), *A. niger* (14.46%), *A. regulosus* (1.87%), *A. sydowi* (3.08%), *A. terreus* (6.09%), *Chaetomium globosum* (7.55%), *Fusarium solani* (2.35*%), Helminthosporium* sp. (5.97%), *Macrophomina phaseolina* (6.88), *Nigrospora sphaericica* 16.69%), *Rhizoctonia solani* (4.19%) and *Rhizopus nigricans* (8.35). The obtained results almost agree with[35,36].

Depending upon their frequent of occurrence the genera and species were grouped as major and minor component include: *Nigrospora sphaericica* (16.6), *Aspergillus niger* (14.46%) and *Alternaria alternata* (11.82%) were the most frequently isolated fungi. Species of *Alternaria* and *Aspergillus* also found in high incidence in seeds of moringa study by [9], which agrees with the obtained result here. Among the so called storage fungi, *Aspergillus* spp. was the most dominant fungi occurred in highest frequent (36.16%). On the other hand, the field fungi (damping-off, root-rot and wilt pathogens), i.e., *Alternaria alternata*, *Fusarium solani, Macrophomina phaseolina* and *Rhizoctonia solani* were also found. This important plant pathogenic fungus is consistently found associated with seeds of different plant species and have the potential cause severe damage[37,38].

Table 2. The frequency occurrence percentage of fungi isolated from moringa seeds recently collected (2014) or collected during seasons of 2011, 2012 and 2013 on PDA agar medium.

Fungi	Recently collected seeds during 2014	Seeds collected at season 2013	Seeds collected at season 2012	Seeds collected at season 2011	Mean
Alternaria alternata	20.00	9.68	9.09	8.51	**11.82**
Aspergillus candidus	10.00	-	3.03	4.26	**4.32**
A. flavus	10.00	3.23	12.12	-	**6.34**
A. niger	-	12.90	15.15	29.79	**14.46**

A. regulosus	-	3.23	-	4.26	**1.87**
A. sydowi	-	3.23	9.09	-	**3.08**
A.terreus	-	6.45	3.03	14.89	**6.09**
Chaetomium globosum	10.00	12.90	3.03	4.26	**7.55**
Fusarium solani	-	-	3.03	6.38	**2.35**
Helminthosporium sp.	-	3.23	12.12	8.51	**5.97**
Macrophomina phaseolina	-	19.35	6.06	2.13	**6.88**
Nigrospora sphaericica	50.00	6.45	6.06	4.26	**16.69**
Rhizoctonia solani	-	6.45	6.06	4.26	**4.19**
Rhizopus nigricans	-	12.90	12.12	8.51	**8.35**

* Tests were run in quadruplicate.
* Counts represent the number of fungi / seed incubated at 28±2ºC for 7 days.

It was observed that the seeds have been collected recently during season of 2014 detected a few genera of fungi reaching only four genera including *,Alternaria, Aspergillus, Chaetomium* and *Nigrospora*, while in the old seeds stored for a year or more the number of genera of fungi reached eight genera. This may be due to the presence of substances in these modern seeds have the ability to prevent fungal growth, while the stored seeds for a year or more could lead to a breakdown of these substances prohibitive for fungal growth.

3.3 Antifungal activity of seed extract:

The results of antifungal activity of moringa seed extracts against some phytopathogenic fungi are shown in Table (3). Moringa seed extracts showed varying degree of antifungal activities against the tested fungal spp. The inhibition zone for all fungi were gradually increased with increase in concentration of the ethanolic and aqueous seed extracts. The ethanolic seed extract was found to be more effective than the water extract. The ethanolic seed extract at 100% cause increase in zone of inhibition in all tested fungi reaching values between 2.70-3.77mm, whereas, at 50% concentration reaching between 1.40-1.93mm. Moreover the ethanolic extract at 100% concentration had the minimum inhibitory effect against *M. phaseolina* (2.70mm) and *F. solani* (2.87mm) compared with 4.83 and 4.57mm of the standard fungicide (+ control) respectively.

The aqueous extract of moringa seeds at 50% concentration showed moderate effect against *M. phaseolina* (0.83 mm) but showed no activity against *Alternaria alternata* and *Aspergillus flavus* (0.0 mm zone inhibition) compared with the standard fungicide (4.37 and 4.90 mm respectively). Whereas, The aqueous extract of moringa seeds at 100% concentration showed moderate activity against all tested fungi (inhibition zone between 0.43-1.33 mm) compared with the positive control. These results are consistent with those obtained by other investigators who found an antifungal activity of moringa plant extracts against several phytopathogenic fungi[6-9,15,16,18,19]. In this respect, Raj, A.J. et al[39]noted that the phytochemical screening of moringa seeds revealed the presence of alkaloids, flavonoids, saponins, erpenoids, steroids, tannins, cardioglycosides, aminoacids and proteins which have antifungal and antibacterial activities.

Table 3. Antifungal activity (zone of inhibition, mm) of ethanolic and aqueous extracts of moringa seed against some phytopathogenic fungi.

Treatment	Conc. (%)	Zone of inhibition (mm)						Mean
		Alternaria alternata	*Aspergillus flavus*	*Chaetomium globosum*	*Fusarium solani*	*Macrophomina phaseolina*	*Rhizoctonia solani*	
Ethyl extract	**0**	0.00N	0.00N	0.00N	0.00N	0.00N	0.00N	**0.00F**
	50%	1.87H	1.93H	1.63H	1.53KL	1.40IJ	1.63HI	**1.67C**
	100%	3.77E	3.53EF	3.23F	2.87IJ	2.70G	3.77E	**3.31B**
+ Control		4.37CD	4.37CD	4.23D	4.57BC	4.83AB	4.37CD	**4.46A**
aqueous	**0**	0.00N	0.00N	0.00N	0.00N	0.00N	0.00N	**0.00F**

extract	**50%**	0.00N	0.00N	0.23MN	0.60IJ	0.83K	0.43LM	**0.35E**
	100%	0.43LM	0.60KL	0.83K	1.33G	1.17J	1.20J	**0.93D**
+ Control		4.37CD	4.90A	4.23D	4.57AB	4.83AB	4.37CD	**4.54A**
Mean		**1.85BC**	**1.92AB**	**1.80BC**	**1.93AB**	**1.97A**	**1.97A**	

3.4 Phytochemical and elemental analyses of moringa seed powder:

The results of the phytochemical and elemental analyses of moringa seed powder are shown in Table (4) and Figs. (2 and 3). The study showed that the seeds of moringa recently collected during 2014 contained phenols and flavonoids at high rates and then decrease during the storage period, as the total phenol was decreased from 7.00 to 3.19mg/g (54.4% decrease) and total flavonoids from 0.697 to 0.550 mg/g (21.09 decrease) in moringa recently collected and stored seeds for 3 years respectively.

Table 4. Moringa seed contents of flavonoids, phenols, protein, micro and macro- nutrients of recently collected during 2014 or produced during seasons of 2011, 2012 and 2013

Seeds stored for	Total phenol (mg/g)	Total flavon-oids (mg/g)	Protein %	Macronutrients %			Micronutrients ppm			
				N	P	K	Fe	Zn	Mn	Cu
Recent during (2014)	7.00	0.697	18.44	5.6	0.94	1.18	259.0	52.1	26.8	6.2
One year storage (2013)	4.92	0.626	16.25	5.5	0.83	1.13	222.5	35.8	21.8	5.1
Two years storage (2012)	4.54	0.601	12.19	5.3	0.91	0.12	204.2	27.5	21.2	4.3
Three years storage (2011)	3.19	0.550	15.94	5.1	0.91	1.11	213.1	29.3	24.8	4.9

The same trend was also found in the presence of proteins, as the highest percent of protein (18.44%) was detected in recently collected seeds and decreased to 15.94 % (13.50% decrease) in stored seeds for 3 years. The presence of phenols, flavonoids and protein are known to be biologically active and therefore aid the antimicrobial activities of moringa. Previous studies have reported that various parts of moringa roots, bark and stem including seeds possess antimicrobial properties[6-9].

Concerning elements analysis in recent and ancient stored seeds, it was found that the recent collected seeds contain largest rates than old stored seeds. The percentage of nitrogen, phosphorus and potassium were high in recently collected seeds reaching 5.6, 0.64 and 1.18 % and reduced to 5.1 , 0.91 and 1.11 % respectively in stored seeds for 3 years.

The study also showed that the storage of seeds reduced the amount of micronutrients, for example Fe was decreased from 259.0 to 213.1 ppm (17.72 % decrease), zinc from 52.1 to 29.3 ppm (43.76% decrease), Manganese from 26.8 to 24.8 ppm (7.46% decrease) and cupper from 6.2 to 4.9 ppm (20.97% decrease) in recent collected and seeds stored for 3 years respectively.

GC/MS analysis of extracts of recent collected moringa seeds and seeds stored for 3 years led to the identification of different compounds. Extract of recent collected seeds during season 2014 contained some compounds varied in their chemical composition and their molecular weight (Table, 5 and Fig. 2). These compounds were differed than that compounds detected in extract of stored seeds for 3 years (Table, 6 and Fig. 3). These variation in seed content could be explained the low infection and high antifungal activity of recent collected seeds than stored seeds.

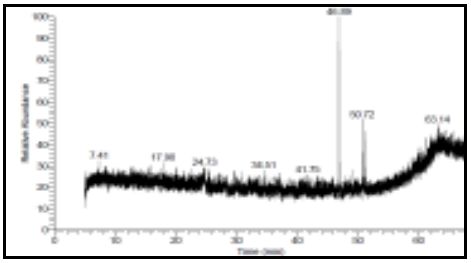

Fig. 2. Main components in moringa seed powder recently collected during season of ,2014 performed by GC/MS.

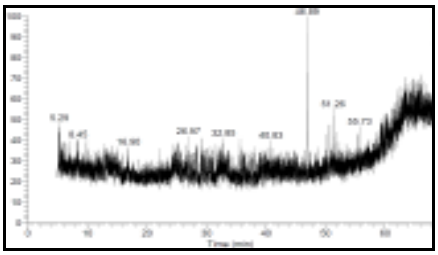

Fig. 3 Main components in moringa seed powder collected during season of , 2011 performed by GC/MS

Table (5): Main components in moringa seed powder recently collected during season of ,2014 performed by GC/MS analysis.

Probability	Compound Name	Molecular Weight	Molecular Formula
57.53	4hydroxy3(2ox o2h1oxa3phe Nanthryl)2(1h)quinolinone	**355**	C22H13NO4
35.78	1Propyne (CAS)	**40**	C3H4
54.55	1,2Propadiene (CAS)	**40**	1,2Propadiene (CAS)
95.76	N,N'Dicyclohexyl1c yano7pyrrolidinylperylene3,4: 9,10tetracar boxylic acid Bisimide	**648**	C41H36N4O4
85.24	4,6Dibromo2[4',5',6'tribromo2' Hydroxyp henoxy]phenol	**592**	C12H5Br5O3
50.35	2(2',6'Dimethoxyphenyl)4,7bis[4'(1",1"dimethyl) Phenoxy]1,10phenanthroline	**612**	C40H40N2O4
82.45	12Methoxy2trimethy lsilyloxy19nor5ápodocarpa1,3,8,11,13pe ntaene	**328**	C20H28O2Si
67.31	Octadecanoic acid,	**356**	C21H44O2Si

Table 6. Main components in moringa seed powder collected during season of 2011 (stored seeds for 3 years) performed by GC/MS analysis.

Probability	Compound Name	Molecular Weight	Molecular Formula
84.58	1[2,4,6tris(trimethyl siloxy) phenyl 3[3,4d i(trimethylsiloxy) phenyl] 2propen1one	**648**	C30H52O6Si5
58.39	Dodecachloro3,4ben zophenanthrene	**636**	C18Cl12
27.92	YGRKKRRQRRRGP VKRRLDL/5	**2598**	N/A (nist_ms)
28.36	1,2Propadiene (CAS	**40**	C3H4
65.88	Dodecachloro3,4ben zophenanthrene	**636**	C18Cl12
50.37	[Tri{Titaniumpentam ethyl cyclo pentadienyl(æoxa)}(æethyl){N,N diethylamino)}]	**697**	C36H59NO3Ti3

64.78	2,7,12,17Tetramethyl 3,5: 8,10:13 ,15 :18 ,20 tetrakis(2,2dimethylpropano) porphyrin	**638**	C44H54N4
76.63	Octadecanoic acid, trimethylsilyl ester (CAS)	**356**	C21H44O2Si

The results of Tables 1and 6) show that stored seeds was highly infected with fungi which might be linked to its phytochemical contents as the phytochemical screening indicated that it possess low phenols, flavonoids, and phytochemical compounds. On the other hand, Tables of 1and 5) show that seeds of moringa recently collected was the least infection which might be linked to its phytochemical contents. This finding was previously reported by[13,40,41] who reported that the antimicrobial activity of *M. oleifera* seed is due to the presence of an array of phytochemicals, but most important due to the activity of saponins, steroids, tannins, glycosides, alkaloids flavonoids and phenols.

Conclusion

This investigation reports that the potential of *M. oleifera* ethanolic and aqueous extracts possesses antifungal activity against phytopathogenic fungi and could be used instead of fungicides to prevent or reduce diseases causing seed to rot and damping-off disease caused by seed borne fungi. Also, it is recommended to use the alcoholic extract of the recently collected moringa seeds to protect seeds of other plants at postharvest in storage.

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