

# Efficacy of Botanical Fungicides against *Curvularia lunata* at Molecular Levels

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## Abstract

*Juniperus procera* and *Avicennia marina* extracts were tested for their antifungal activity against *Curvularia lunata* isolated from rice (*Oryza sativa* L.). Eextracts were able to suppress *C. lunata* at high concentration 3 mg/ml. *J. procera* extract retarded the growth *C. lunata* by 88.42% while *A. marina* extract was less effective (37.50%). Nucleic acids content of *C. lunata* was reduced by both plant extracts especially *J. procera* extract in a distinct reverse proportion relationship when compared with the untreated samples. The antifungal effect of *J. procera* and *A. marina* extracts were explored at the molecular level, using random amplification of polymorphic DNA (RAPD). The results demonstrated polymorphic banding pattern. Secondary metabolites analysis of *C. lunata* revealed that both plants extracts were capable of blocking biosynthesis of some secondary metabolites including curvularic acid and lutein.

**Keywords:** Efficacy; Botanical fungicides; suppression; *Curvularia lunata*; Molecular levels

## Introduction

About 738 million metric tons rice are produced annually [1], therefore Gautam et al. [2] suggests that it is necessary to check the rice grains before allowing distribution for public use. Different fungi are significant destroyers of seeds during storage and crops at seedling stage. They are also remain dormant in seed and transmit to seedlings and mature plant showing different symptoms [3]. Fungi including, *Curvularia oryzae*, and *C. lunata*, have been isolated from seeds of different varieties of rice [4-6]. *Curvularia phytopathogenic* species produce spots in grains and seeds, as well as damage to plant leaves, their species are saprophytes, occur mostly in tropical and subtropical areas, and are isolated from soil, air, organic matter, plants and animals, including humans [7]. This genus of filamentous fungi colonizes soil and vegetation and spreads by airborne spores. Some of the 40 *Curvularia* species are phytopathogens. Plant diseases range from seedling failure to leaf blight, [8]. Curvularial growth on stored grain, thatch, and other dead plant material looks like smudges of blackish dust. Culture filtrate of *Curvularia lunata* caused reduction in seed germination, radicle, plumule length and low vigour index of *Trigonella foenumgraecum* [9].

It is now realized that chemical fungicides cause serious environmental problems and are toxic to non-target organisms [10-13]. The toxic effect of synthetic chemicals can be overcome, only by persistent search for new and safer pesticides accompanied by wide use of pest control methods, which are eco-friendly and effective. Plant metabolites and plant based pesticides appear to be one of the better alternatives as they known to have minimal environmental impact and danger to consumers in contrast to the synthetic pesticides [14,15]. Rahman [16] observed that bishkatali, garlic, ginger and neem extract were effective against seed-borne *Curvularia lunata*. Garlic extract was superior in terms of reducing seed-borne infections by *Alternaria spp.*, *Bipolaris sorokiniana*, *Curvularia lunata*, *Fusarium spp.* of wheat to other extracts followed by ginger and neem [17,18]. Methanolic extracts of *Bridelia montana* and *Scoparia dulcis* showed the high antifungal activity against *C. lunata* and recorded minimum inhibitory concentration below 50 mg/ml. The overall reports provide promising

baseline information for the potential use of the crude extracts from medicinal plants in the treatment of fungal diseases caused by *Curvularia lunata* [19]. The efficacy of leaf extracts of *Gliricidia sepium*, *Tithonia diversifolia*, *Phyllanthus amarus* and *Morinda lucida* were assessed *in vitro* [20] to control *C. lunata*. The extracts of the four plants suppressed the growth of *C. lunata in vitro*. Mangroves are medicinal plants and extracts from different parts of them are widely used through the world. These are considered as rich sources of steroids, triterpenes, saponins, tannins, alkaloids and flavonoids. Antiviral, antibacterial and antifungal activity of the extracts from these plants have been shown in former studies [21,22]. *A. marina* and *A. officinalis* were used as test plants due to the presence of much evidence that prove their therapeutic value against microbial infections [23]. Also preliminary studies have been demonstrated that the mangrove plant extracts have antibacterial activity against pathogenic bacterial strains; *Staphylococcus sp.*, *E. coli* and *Pseudomonas sp.* [24]. *Juniperus* species have been extensively investigated as a source of natural products with potential antibacterial, antifungal and insecticidal activities [25-27]. Mariana and Camelia, [28] reported that Juniper oil has inhibition action against *Aspergillus niger*, *Fusarium oxysporum*, *Monascus purpureus* and *Penicillium hirsutum*. The aim of this study was to determine antifungal activity *J. procera* and *A. marina* extracts as safe alternative for controlling *C. lunata* and their mechanisms on molecular levels.

## Material and Methods

### Isolation and identification

Stored grains of rice (*Oryza sativa* L.) were surface sterilized

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with 0.1% mercuric chloride for two minutes followed by washings with sterilized water. The surface sterilized grains were transferred on three layered filter paper beds in 9 cm diameter sterilized Petri plates containing Czapek-Dos growth medium. The growing fungus was identified according to the cultural characteristics and spore morphology [29] as *Curvularia lunata*.

### Treatments

*Juniperus procera* and *Avicennia marina* leaves were collected from Jazan region, KSA. Fresh leaves (500 g) of plants were air-dried at room temperature, ground into powder using an electric grinder, then extracted with methanol in a Soxhlet. The solvent was removed using rotary evaporator under reduced pressure at temperature below 50°C. The resulting crude extracts were stored at 4°C in dark until used. Carbomar (Methyl Benzimidazol-2-ylcarbamate) from (Gyangoetnal, China) was used as chemical fungicide.

### Anti-fungal assay

Poisoned food technique was used. Potato dextrose agar medium (PDA) supplemented with different concentrations of each plant extract and carbomar were prepared separately. About 25 ml of the growth medium was poured into each petri-dish and allowed to solidify. Five mm disc of 5-day-old culture of the *Curvularia lunata* was placed at the center of the petridish and incubated at 27°C for 7 days, the growth was measured in millimeter. For each treatment three replicates were maintained. PDA medium without the methanolic extract served as control [30]. The toxicity each treatment in terms of inhibition percentage of mycelial growth was calculated by using the formula:

$$\text{Inhibition(\%)} = \frac{D_c - D_t}{D_c} \times 100$$

Where  $D_c$ =average increase in mycelial growth in untreated samples,  $D_t$ =Average increase in the treated samples [31].

### Secondary metabolites detection

After 10 days of incubation period, the growth medium (PDA broth containing 1 and 2mg/ml of *J. procera* and *A. marina*; 5 and 25 µg/ml of Carbomar) of *C. lunata* containing extracellular metabolites was extracted twice with Chloroform/methanol (2:1 v/v), then concentrated and separated using High-Performance Thin-Layer Chromatography (HPTLC) techniques. Twenty microliters of the samples, with or without treatment, were applied to HPTLC plates (10 cm × 10 cm, 0.2 mm silica gel Merck 60 F 254 precoated plate; Merck Darmstadt, Germany) using CAMMAG LINOMAT 5 application system. The TLC plates were eluted for the detection of extracellular metabolites in toluene/ethyl-acetate/90% formic acid 5:4:1 (TEF). Once the runs had finished, the plates were observed under visible and ultraviolet at 254 and 365 nm illumination. Griseofulvin dissolved in chloroform/methanol 2:1 was used as standard in all cases and relative retention factor value ( $R_f$  value) to griseofulvin were calculated as  $R_{fg}$ . To identify the metabolites the absolute  $R_f$  and the relative  $R_f$  to griseofulvin ( $R_{fg}$ ) were measured. Secondary metabolites were identified by descriptions in literature and comparison with the available standard was made [32-35]. Retention factor value ( $R_f$  value): The distance that the spot of a particular compound moved up on the TLC plate relative to the distance moved by the solvent front is called the retention factor or  $R_f$  value. The  $R_f$  values of individual secondary metabolites were calculated by following,  $R_f$ =Distance traveled by the compound/Distance travelled by the solvent. Qualitative determination of secondary metabolites

were done by comparing the  $R_f$  values calculated using CAMMAG TLC scanner at Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University Cairo, Egypt.

### RAPD analysis

DNA from each treated and untreated *C. lunata* mycelia was extracted using DNeasy kit (Qiagen-Germany). PCR amplification was conducted using 5 random primers listed in Table 1 [36]. The amplification was performed in a thermal cycler programmed as follow: Cycle-1: 94°C for 10 minute, Cycle-2 : 97°C for 15 minute, 36°C for 1 minute, 72°C for 2 minute Repeat for 40 times, Cycle-3: 72°C for 10 minute and Cycle-4: 4°C for 30 minute. PCR amplification products were separated by electrophoresis using 1.5% agarose gel and 100 bp ladder. The products were visualized and photographed under UV transilluminator.

The banding patterns of RAPD were scored using Gel analyzer 2010a software and data fed to the computer as 1 and 0 for the presence and absence of bands, respectively. Pair-wise comparisons of genotypes, based on the presence or absence of monomorphic and polymorphic fragments, were used to generate percentage of polymorphism.

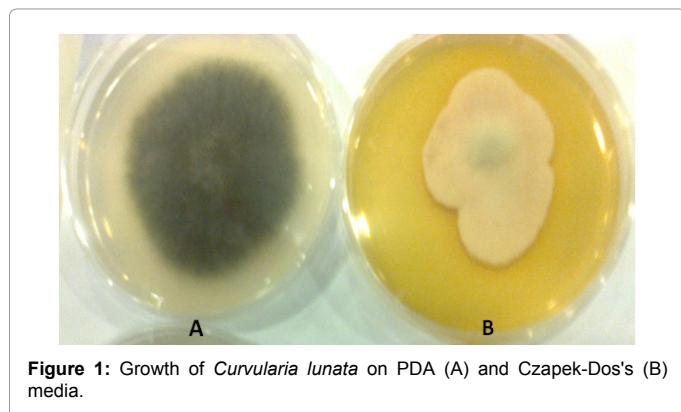
### Results and Discussion

*Curvularia lunata* was found associated with the contaminated seeds of rice (*Oryza sativa* L.). Under microscopic examination, Conidia were smooth-walled, olivaceous brown, end cells somewhat paler, conidia obovoidal to broadly clavate, curved at the subterminal cell, 25-30 × 10-14 µm. Conidiophores were erect, septate, unbranched, flexuose in the apical part, with flat, dark brown scars., 3-septate. Morphological characteristics of the isolated fungus were in agreement with Ellis, [29]. Earlier study analyzed 25 rice samples that were contaminated with one or more fungal genera like *Aspergillus*, *Fusarium*, *Curvularia*, *Cladosporium* and *Alternaria* [37]. *Curvularia trifolii*, *C. lunata* and *C. brachyspora* were isolated from 40 different paddy samples [38]. Isolated *Curvularia lunata* colonies showed morphological variation on the two used media (Czapek-Dox and potato dextrose agar media). *C. lunata* colony appeared light-brown to grey on Czapek-Dox medium while it turned to buff light when grown on PDA medium (Figure 1). The fungus failed to sporulate on PDA medium but heavy sporulation occurred on Czapek-Dox agar. The differences in media composition particularly, the carbon and nitrogen sources might be the cause of these phenomena. Lanisnik Rizner and Wheeler, [39] and Lanisnik Rizner and Romih, [40] observed that *C. lunata* was dark brown when grown in malt extract broth, however, it remained white or very pale brown when grown in yeast nitrogen base broth medium.

*C. lunata* was completely inhibited by 50 µg/ml of chemical fungicide Carbomar. Similar results were obtained by Shikha et al. [41] who used Carbendazim (10 mg/ml) to inhibit *C. lunata* growth. Topsin and mencozeb suppressed the growth of *Curvularia* sp. by 50% [42]. Even though effective and efficient control of seed borne pathogenic fungi can be achieved by the use of synthetic fungicides, they cannot be applied to grains because of their toxicity [12,43]. Thus, there is a need for safe, nontoxic, eco-friendly and cheap alternative approaches to store

Primer code	Nucleotide sequence 5'-3'
P1	AGGAGGACCC
P2	ACGAGGGACT
P14	CCACAGCAGC
PE7	AGATGCAGCC
PE20	AACGGTGACC

Table 1: Ten-mer random primers.



**Figure 1:** Growth of *Curvularia lunata* on PDA (A) and Czapek-Dos's (B) media.

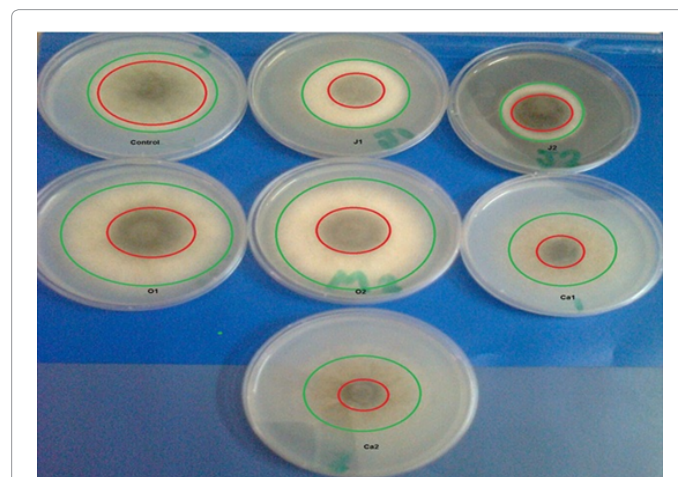
grains/cereals for human consumption. Exploration of safe alternative antifungal agents, especially the plant extracts has intrinsic worth. Plant extracts as potential antifungal compound has been explored against several fungal species. In this study, two plants (*J. procera* and *A. marina*) extracts reduced *C. lunata* mycelium growth (Table 2). Although none of these plants extracts inhibited the mycelial growth 100%, these plants have been reported to possess antifungal properties against *C. lunata*. *J. procera* extract was more effective against *C. lunata* than *A. marina*. *C. lunata* growth was inhibited by 88.42 and 37.50 % when growth medium was treated with *J. procera* and *A. marina* extracts respectively. Recently, Abdelghany [27] reported the antifungal effect of *J. procera* crude extract against growth of *Aspergillus flavus* and *Fusarium oxysporum*. Surprisingly, *A. marina* extract at low concentrations induced the vegetative growth of *C. lunata* (Figure 2), but it suppressed the fungal sporogenesis. This may be due to *A. marina* extract component(s) reduced the activity of sporogenesis enzymes which saved energy for mycelial growth. Antifungal activity of *A. marina* was investigated Nayak et al., [44] who concluded that the leaf extract has both antifungal and antibacterial activities. Antifungal metabolites of *A.marina* include alkaloids, flavonoids and related compounds, fatty acids, oxygen heterocyclics, proanthocyanidins, quinones, stilbenes, terpenoids and triterpenoid, saponins [45].

Natural antifungal agents can be potential exploited in controlling the growth of fungi consequently inhibiting secondary metabolites production [46,47]. Therefore in recent years, much attention has been given to the preservation of grains by natural products so that the growth and mycotoxin production may be effectively retarded. *Curvularia lunata* secondary metabolites qualitative profile (Table 3 and Figure 3) indicates the ability of *J. procera* and *A. marina* extracts and carbommar to alter its metabolic pathways. *Curvularia* sp. secondary metabolites profile has been investigated by Trisuwan and his team [48], curvulapyrone, curvulalide and curvulalic acid, modiolides A and B, pyrenolide A, stagonolide E, mycoepoxydiene, and deacetylmycoepoxydiene were identified. *J. procera* extract low concentration (1.0 mg/ml) was capable of blocking the biosynthesis

of curvulapyrone and radicinin while higher concentration (2 mg/ml) blocked the synthesis of lunatin and curvulalic acid in addition to curvulapyrone and radicinin. Deacetylmycoepoxydiene, mycoepoxydiene, cytochalasin B, radicinin and curvulapyrone were not synthesised by *C. lunata* in both media treated with low and high concentration of *A. marina* extract, while lunatin was not synthesised only in *A. marina* high concentration media. Except of cytoskyrins, all investigated metabolites were blocked by both carbommar concentrations. *C. lunata* produces cytochalasin B [49], that is a mycotoxins acts on the cytoskeleton and may show other bioactivities [50]. *A. marina* extract and carbommar inhibited cytochalasin B.

Al-Qurainy and Abdel-Megeed [51] stated that some antifungal agents have the capacity to alter the genetic material. This statement pushed this research to investigate nucleic acids content in both treated and untreated *C. lunata* samples (Table 4). Both plants extracts induced an increase in total DNA and RNA content of *C. lunata* especially at relatively low concentrations 1 and 2 mg/ml growth medium. On the other hand, it was found that DNA and RNA content decreased with the increase plant extracts specially with using *J. procera*. Carbommar sharply decreased the nucleic acids content of *C.lunata* compared with the control and plant extracts used.

Alternation in growth, sporulation, secondary metabolites pathways and nucleic acids content of *C. lunata* treated with *J. procera* extract, *A. marina* extract, and carbommar drive this study to investigate the potential genotoxic effects of the *J. procera* and *A. marina* extracts at molecular level using RAPD analysis. Two levels in the form of two different concentrations were tested for each extract against wild type (untreated sample). Carbommar treated samples were the positive



**Figure 2:** Growth and sporulation of *C. lunata* on treated Czapek-Dox agar medium (J1, J2: 1 and 2 mg/ml *J. procera* extract respectively; M1, M2: 1 and 2 mg/ml *A. marina* extract respectively; Ca1, Ca2: 5 and 10 µg/ml Carbommar respectively).

Plant extract Concentration (mg/ml)	<i>J. procera</i>		<i>A. marina</i>		Carbommar		
	Colony radius (mm)	Inhibition (%)	Colony radius (mm)	Inhibition (%)	Concentration (µg/ml)	Colony radius (mm)	Inhibition (%)
0.0	5.60 ± 0.10	0.00	5.60 ± 0.10	0.00	0.00	5.60 ± 0.10	0.00
1.0	5.20 ± 0.10	41.89	6.70 ± 0.12	0.00	5.00	4.30 ± 0.02	23.21
1.5	3.90 ± 0.12	48.64	6.70 ± 0.15	0.00	10.00	3.20 ± 0.14	42.85
2.0	3.20 ± 0.15	59.86	5.50 ± 0.10	8.00	25.00	1.70 ± 0.12	69.64
2.5	2.50 ± 0.12	76.08	4.30 ± 0.12	23.21	50.00	0.00 ± 0.00	100
3.0	0.80 ± 0.06	88.42	3.50 ± 0.10	37.50	100	0.00 ± 0.00	100

**Table 2:** Effect of plant extracts and Carbommar different concentrations on the growth of *Curvularia lunata*.



Secondary metabolite	Control	<i>J. procera</i> extract concentration (mg/ml)		<i>A. marina</i> extract concentration (mg/ml)		Carbomar Concentration (µg/ml)	
		1	2	1	2	5	25
Cytoskyrins	+	+	+	+	+	+	+
Lunatin	+	+	-	+	-	-	-
Curvulapyrone	+	-	-	-	-	-	-
Curvulalide	+	+	+	+	+	-	-
Curvulalic acid	+	+	-	+	+	-	-
Radicinin	+	-	-	-	-	-	-
Cytochalasin B	+	+	+	-	-	-	-
Mycoeoxydiene	+	+	+	-	-	-	-
Deacetylmycoeoxydiene	+	+	+	-	-	-	-

Table 3: *C. lunata* secondary metabolites profiles at different concentration of plant extracts and Carbomar.

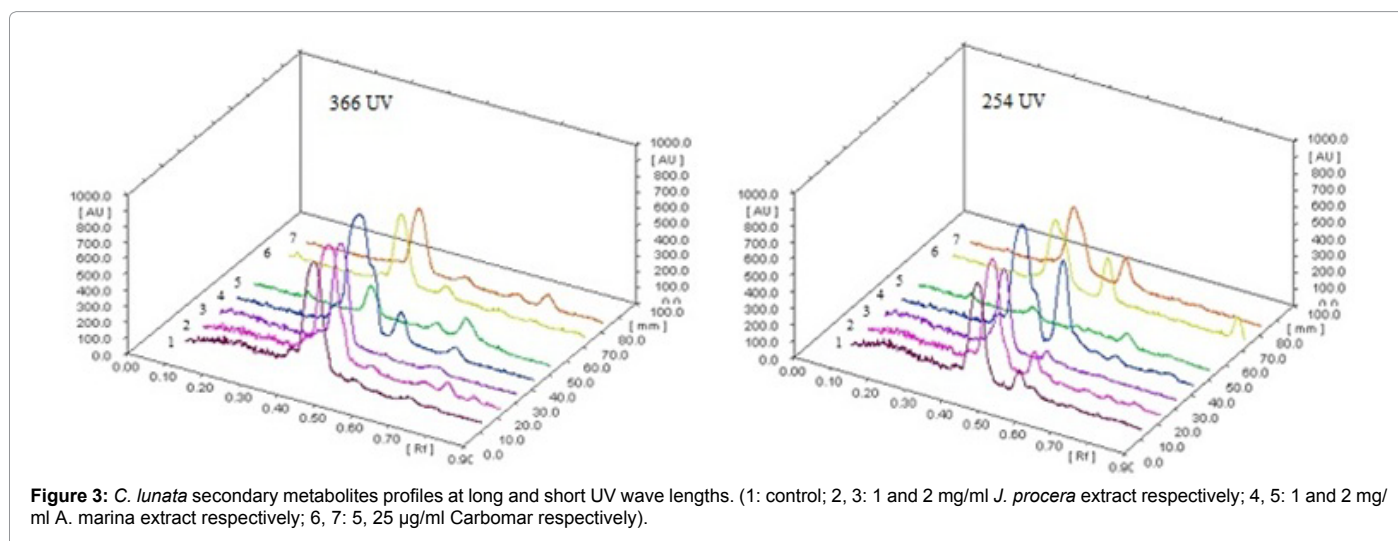


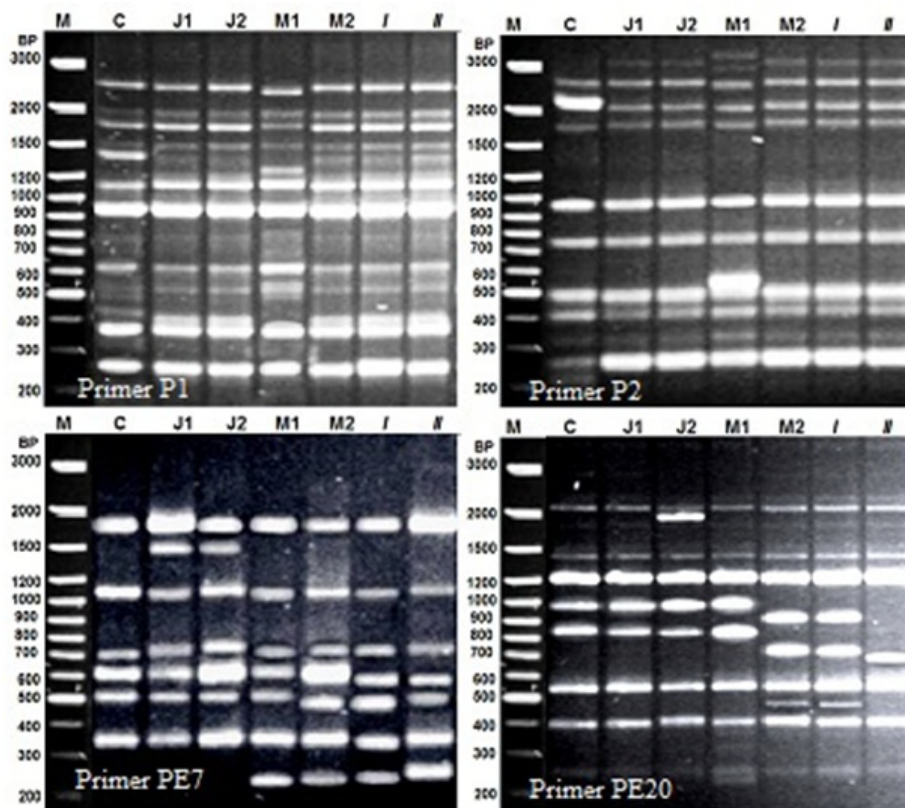
Figure 3: *C. lunata* secondary metabolites profiles at long and short UV wave lengths. (1: control; 2, 3: 1 and 2 mg/ml *J. procera* extract respectively; 4, 5: 1 and 2 mg/ml *A. marina* extract respectively; 6, 7: 5, 25 µg/ml Carbomar respectively).

Extract Conc. (mg/ml)	<i>J. procera</i> extract		<i>A. marina</i> extract		Carbomar (µg/ml)	Carbomar	
	DNA	RNA	DNA	RNA		DNA	RNA
0.0	8.75 ± 0.26	60.50 ± 0.10	8.75 ± 0.26	60.50 ± 0.10	0.00	8.75 ± 0.26	60.50 ± 0.10
1.0	10.20 ± 0.15	65.25 ± 0.12	9.30 ± 0.16	75.25 ± 0.10	5.00	4.05 ± 0.20	45.00 ± 0.20
1.5	9.10 ± 0.25	69.10 ± 0.20	11.50 ± 0.20	79.10 ± 0.09	10.0	4.15 ± 0.20	35.00 ± 0.10
2.0	6.15 ± 0.10	40.22 ± 0.20	10.00 ± 0.05	60.22 ± 0.10	25.0	No fungal growth	3.05 ± 0.15
2.5	6.25 ± 0.15	40.30 ± 0.25	7.05 ± 0.10	50.30 ± 0.20	50.0		
3.0	4.30 ± 0.20	40.00 ± 0.20	5.15 ± 0.26	42.00 ± 0.08	100		

Table 4: Effect of different concentrations of *J. procera*, *A. marina* extracts and Carbomar on DNA and RNA concentrations (mg/g mats dry weight of mass) of *Curvularia lunata*.

control. RAPD analysis was extensively used as molecular analysis tool for *Curvularia sp.* [52-55]. Five 10-mer random primers were tested for RAPD analysis. Four primers (P1, P2, PE7 and PE20) produced considerable bands (Figure 4) while the primer P14 produced no distinct bands. A total of 52 amplified fragments were observed, 33 of them were monomorphic bands while 19 were polymorphic that reflect high degree of polymorphism (36.5%) among all samples. Comparison of each treatment with the untreated sample (wild type) showed that *C. lunata* treated with *A. marina* extract showed the highest degree of polymorphism (32%) among its 50 amplified fragment which recommend that *A. marina* has high mutagenic efficacy; followed by the carbomar which induced 14 polymorphic band (29.2% polymorphism) from 48 total amplified fragments. These molecular markers can demonstrate similarities and dissimilarities between different isolates of same species even when a morphological description is severely limited. *J. procera* comes finally as its treated *C. lunata* samples showed

the lowest degree of polymorphism with 19% polymorphism and 42 amplified fragments (Table 5). Studying the effect of concentration on mutagenic rate in terms of percentage of polymorphism indicated that for *J. procera* and *A. marina* it was clear that there is a proportion between the concentration of the treatment and the percentage of polymorphism in the treated *C. lunata* samples. *A. marina* extract low concentration result in 35 monomorphic fragments and 8 polymorphic that represent 18.6% of polymorphism in the treated fungal samples. The high concentration showed higher level of polymorphism with 33 monomorphic fragments and 12 polymorphic fragments (26.7% polymorphism). The same phenomena was noticed while studying *J. procera* different concentrations (Table 6). This observation gives good evidence to the ability of these plant extracts and chemical fungicide Carbomar to induce mutation as a result of compromising at least one nucleotide as revealed by the disappearance of many genetic bands and change in restriction endonucleases sites as compared with untreated



**Figure 4:** Agarose gels for primers P1, P2, PE7 and PE20 amplification products. (J1, J2: 1 and 1.5 mg/ml *J. procera* extract respectively; M1, M2: 1 and 1.5 mg/ml *A. marina* extract respectively; I, II: 5 and 10 µg/ml Carbomar).

Treatment	Amplification Products	Ten mer random primers codes				Total
		P1	P2	PE7	PE20	
<i>J. procera</i>	TAF	14	11	7	10	42
	Poly%	21.43	18.18	14.29	20.00	19.05
	Mono%	78.57	81.82	85.71	80.00	80.95
<i>A. marina</i>	TAF	17	12	9	12	50
	Poly%	35.29	25.00	22.22	41.67	32.00
	Mono%	64.71	75.00	77.78	58.33	68.00
Carbomar	TAF	15	11	9	13	48
	Poly%	26.67	18.18	22.22	46.15	29.17
	Mono%	73.33	81.82	77.78	53.85	70.83

**Table 5:** Amplification products polymorphism of treated *C. lunata* with plant extracts and chemical fungicide carbomar. TAF: total amplified fragments; Poly: Polymorphic fragments; Mono: Monomorphic fragments.

Treatment	Conc. (mg/ml)	P1		P2		PE7		PE20		Total	
		TAF	Poly%	TAF	Poly%	TAF	Poly%	TAF	Poly%	TAF	Poly%
<i>J. procera</i>	1.00	14	21.43	11	18.18	7	14.29	9	0.00	41	14.63
	2.00	14	21.43	11	18.18	7	14.29	12	41.67	44	25.00
<i>A. marina</i>	1.00	15	26.67	12	25.00	7	14.29	9	0.00	43	18.60
	2.00	15	26.67	11	18.18	7	14.29	12	41.67	45	26.67
Carbomar	0.5	15	26.67	11	18.18	7	14.29	13	46.15	46	28.26
	2.5	15	26.67	11	18.18	9	22.22	11	36.36	46	26.09

**Table 6:** Polymorphism of *C. lunata* treated with plant extracts and chemical fungicide carbomar.

*C. lunata*. Generally in all cases, polymorphisms were due to the loss and/or gain of amplified bands in the treated samples compared with the control. This result s were agreement with Sameer [56] in their study on the effect of *Calotropis procera* latex on *Aspergillus terreus*. Our findings support this claim that DNA polymorphisms detected by

RAPD can be considered as a powerful biomarker assay for detection of the level of DNA damage in various treated *C. lunata* and to extracts of *J. procera* and *A. marina*, as suggested by similar results obtained by Hajar and Nehad [57] in their study on the effect of *Moringa peregrine* extract on bacteria and fungi.

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