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Associated mycoflora with fresh and market Bael (*Aegle marmelos* Corr.) roots and their effects of total alkaloids and glycosides amounts

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ABSTRACT

Total 20 and 15 fungal species were isolated from fresh and market root samples of drug *Aegle marmelos* Corr. In the fresh samples, *F. solani* with 22.25% and in market samples *A. niger* with 26.97% recorded in high percentage incidence. Root samples were stored under different 30, 50, 75, 96 and 100 % relative humidity and different incubation days 15, 30, 45 and 60 days. Maximum growth and occurrence incidence of fungi were observed under high relative humidities 75, 96 and 100% RH and long storage periods 45 and 60 days. Quantitative estimation of alkaloids and glycosides in association with isolated fungi was done. Rate of deterioration of chemical constituents in the samples were noted at above 75% RH. Analysis of variance also showed that the effect of relative humidity and incubation days on biodeterioration of these chemical constituents were significant at 5% level of significance.

Key words: *Aegle marmelos*, fungi, relative humidity, incubation days

INTRODUCTION

The plant *Aegle marmelos* is commonly called as "Bael" belonging to family "Rutaceae". Root bark is used in intermittent fever and as fish poison, as a remedy for palpitation of heart and melancholia. Juice of the bark with a little cumin in milk is valued as remedy for poverty of seminal fluid. Alcoholic extract of roots having hypoglycaemic activity Bael is reported to contain a number of coumarins, proteins, carbohydrates, phenols, alkaloids, sterols and essential oils. Roots, barks and fruits are hypoglycaemic, astringent and febrifuge and they are antidiarrhoeal and antivenin (Warrier *et al.* 1995). Gond *et al.* (2007) isolated endophytic fungal from this plant. Practices used in harvesting, handling, storage, production and distribution make medicinal plants subject to contamination by different fungi, which may be responsible for spoilage and production of mycotoxins. However, there is scanty information regarding the deterioration of alkaloids and glycosides of medicinal plants. Therefore, in this present study aimed to study mycoflora associated with Bael roots under influence of different relative humidity (RH) and different incubation days in fresh and storage conditions, and also observed the changes in total alkaloids and glycosides amounts.

MATERIALS AND METHODS

The fresh roots of drug *A. marmelos* were collected in healthy, flowering and fruiting conditions from different localities of Maharashtra, India. Market drug samples were also collected from various Shopkeepers and Kashtausdhis. For avoiding aerial contamination, samples were brought to the laboratory in polyethylene bag. Agar plate method and Blotter test and as recommended by International Seed Testing Association (1966) were done for isolation of mycoflora associated with roots. Then samples sterilized with 2% sodium hypochlorite solution and thoroughly washed with sterilized distilled water. For evaluation of biodeterioration of alkaloids and glycosides contents related to mycoflora, the root samples were stored in small muslin clothes at 30, 50, 75, 96 and 100 % RH for 90 days in the room temperature. The root samples were taken out internal 15, 30, 45, 60, and 75 and 90 days, thoroughly washed with distilled water and plated in Petri plates. The percentage incidence of mycoflora was

recorded from first day to 60th day of storage. Fungi were identified by using different keys, such as Raper and Thom (1949), Barnet and Hunter (1972); Thom and Raper (1945); Both (1971) and Nelson *et al.* (1983). Some parts of washed root samples were dried in oven and powdered by grinder and were used for biochemical analysis by following methods of Harborne (1973) for alkaloids and Kokate *et al.* (2002) for glycosides.

Simple correlation were run between selected parameters using Statistical Package for Social Science (SPSS) software in which statistical significance was determined at 0.05 % probability levels.

RESULTS AND DISCUSSION

Results of mycoflora associated with fresh and market samples:

Total 20 and 15 different fungi species were isolated from fresh and market samples of *Aegle marmelos*. Total 20 different fungi were isolated from *Aegle marmelos* roots in genuine and under storage. *F. solani*, *F. oxysporum*, *F. lateritum*, *F. acuminatum*, *F. sambucinum*, *A. flavus*, *A. niger*, *Monilia sitophila*, *Sordaria fimicola*, *Theilavia terricola*, *Rhizopus oryzae*, *Didymostilbe* sp., *Acremonium* sp., *Trichoderma* sp., *Curvularia lunata*, *Nigrospora oryzae*, *Chaetomium globosum*, *Achlya* sp., *Papulaspora immerse* and *Aphanomyces* sp. were isolated from roots of Bael in genuine condition, in percentage incidences of 22.25, 8.26, 2.66, 0.66, 4.96, 5.86, 3.06, 1.96, 1.13, 2.43, 1.96, 5.76, 8.19, 3.58, 2.42, 7.97 and 2.89%, respectively (Graph1). From market roots of this drug, total 15 species of fungi were found which include: *Aspergillus niger*, *A. terreus*, *A. ochraceus*, *A. flavus*, *A. fumigatus*, *A. parasiticus*, *A. tamarii*, *Curvularia lunata*, *Chaetomium globosum*, *Ch. spirale*, *Helminthosporium* sp., *Rhizopus oryzae*, *Trichoderma* sp., *Papulaspora immerse* and *Penicillium* sp. with percentages incidences of 26.97, 8.43, 3.33, 9.06, 11.14, 2.81, 1.85, 2.08, 1.97, 0.93, 0.62, 8.54, 13.22, 8.12 and 0.83%, respectively (Graph2).

Results of effect of relative humidity and incubation days on fungal growth:

The fresh roots of drug Bael stored at various relative humidities 30, 50, 75, 96 and 100% RH. After 15 days, samples stored under different RH and then was taken out and number of isolated fungi counted and percentage incidence of fungi calculated (table 1). Under 30% RH, after 30 days of storage percentage incidence of total fungi recorded 0.34%. It increased after 60th days of incubation to 0.75%. In the case of 50% RH the percentage incidence of fungi from 15th days to 60th days increased from 0.61% to 4.54%. Similarly in 75% RH after 15, 30, 45 and 60th days of storage they showed the percentage incidences 1.2, 2.78, 5.03 and 7.19%, respectively. Under 96% RH the percentage incidence after 15 days of incubation was 2.71% while after 30, 45 and 60th days of incubation increased to 5.37%, 7.94% and 11.50%. Lastly under 100 % RH the percentage incidence observed 4.32%, 8.44%, 13.13% and 17.98% incidence after 15, 30, 45 and 60 days of storage period respectively.

The market root samples of Bael showed various percentage incidences (Table2). Under 30% RH, after 15 days of storage total percentage incidence of fungi recorded 0.28% that increased to 1.83% on 60th of incubation. In the case of 50% RH, total percentage incidence of fungi from 0.42% increased to 3.52% from 15th days to 60th days of incubation. Under 75% RH, percentage incidence observed 0.845% after 15 days of incubation increased to 8.35% on 60th days of incubation. Similarly, in the cases of 96 and 100% RH, total percentage incidence of fungi, after 15 days observed 1.22 and 2.67% increased to 12.74 and 18.15 % on 60th days of incubations.

Results of the deterioration of total alkaloids and glycosides amounts:

The control of fresh and market samples of this drug contained 16.56 and 16.62% total alkaloids. The samples were stored at 30 % RH showed the deterioration of alkaloid after 30 days were 16.56 % and 16.61% and after 60 and 90 days deteriorated to 16.26, 16.37% and 15.96, 15.92% in fresh and market samples respectively. In case of 50% RH, total alkaloid amount after 30, 60 and 90 days of incubation recorded 16.53, 16.60; 16.03, 16.10% and 15.91, 15.83% in fresh and market samples. Maximum reduction in total alkaloid amount observed in case of 96 and 100 %RH, the samples stored at 15, 45, 75 and 90 days showed, 16.56, 16.56%; 16.02, 15.98%; 15.87, 15.84% and 15.84, 15.80% in samples which collected in genuine condition. In case of market samples deterioration of total alkaloids showed more reduction. Under 96 and 100 % RH, after 15, 45, 75 and 90 days of incubation total amount of alkaloid observed 16.59, 16.58%; 16.27, 16.20%; 15.78, 15.76% and 15.71, 15.66%, respectively (Table 3).

The genuine roots of *A. marmelos* stored under 30, 50, 75, 96 and 100% RH. The control samples of *A. marmelos* contained 9.53%, total glycosides and treated sample stored under 30 and 50 % RH showed 9.48 and 9.41% total glycosides after storage of 15 days of incubation, after 30, 60 and 90 days of storage it showed the deterioration 9.4, 9.18, 8.74%; 9.31, 9.001, 8.32% respectively. Similarly, in case of 75 % RH also observed the reduction of glycosides to 8.32% after 90 days of storage. In cases of 96 and 100% RH the reduction in the value of glycosides observed to 9.3, 9.15, 8.78, 8.31% and 9.53, 9.09, 8.61, 8.2% after 15, 30, 60 and 90 days of storage.

Table1: Percentage incidence of fungal isolated from the root of *Aegle marmelos* (Fresh sample) stored at various relative humidity

Mycoflora	con	30%				50%				75%				96%				100%			
		15	30	45	60	15	30	45	60	15	30	45	60	15	30	45	60	15	30	45	60
<i>F. solani</i>	-	0.34	0.41	0.61	0.75	0.55	0.75	1.03	1.23	0.68	1.03	1.16	1.37	0.82	1.23	1.44	1.92	1.03	1.51	1.92	2.47
<i>F. oxysporum</i>	-	-	-	-	-	-	-	0.27	0.41	0.13	0.34	0.48	0.61	0.27	0.55	0.75	1.031	0.41	0.75	1.031	1.23
<i>F. lateritum</i>	-	-	-	-	-	-	-	-	0.34	-	0.27	0.48	0.61	0.48	0.61	0.75	1.031	0.48	0.82	1.10	1.23
<i>F. acuminatum</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.20	0.34	0.48	-	0.27	0.55	0.82
<i>F. sambucinum</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.13	-	0.06	0.20	0.27
<i>A. flavus</i>	-	-	-	-	-	-	-	-	-	0.06	0.20	0.27	0.34	0.13	0.41	0.48	0.61	0.20	0.48	0.75	1.03
<i>A. niger</i>	-	-	-	-	-	-	-	-	0.27	-	0.13	0.34	0.48	0.20	0.41	0.61	0.75	0.34	0.48	0.82	1.031
<i>Monilia sitophila</i>	-	-	-	-	-	-	-	0.13	0.20	-	-	0.27	0.41	-	-	-	0.48	-	0.34	0.48	0.75
<i>Sordaria fimicola</i>	-	-	-	-	-	-	-	-	0.20	-	-	-	0.20	-	-	0.20	0.34	-	-	0.41	0.61
<i>Theilavia terricola</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06	0.20	0.06	0.06	0.27	0.48
<i>Rhizopus oryzae</i>	-	-	-	-	-	-	-	-	0.27	-	-	-	0.34	-	-	0.06	0.34	0.13	0.41	0.48	0.61
<i>Didymostilbe</i> sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06	0.13	0.20	0.06	0.20	0.27	0.41
<i>Acremonium</i> sp.	-	-	-	-	-	-	-	-	0.13	-	-	0.20	0.27	-	-	0.27	0.41	0.06	0.20	0.34	0.55
<i>Trichoderma</i> sp.	-	-	-	-	-	-	-	-	-	-	-	-	0.13	-	-	0.13	0.27	-	0.20	0.48	0.75
<i>Curvularia lunata</i>	-	-	-	-	-	-	-	0.20	0.34	-	0.20	0.48	0.61	0.27	0.41	0.55	0.031	0.41	0.55	0.75	0.96
<i>Nigrospora oryzae</i>	-	-	-	-	-	-	-	0.34	0.48	0.20	0.34	0.55	0.61	0.34	0.55	0.75	0.96	0.41	0.61	0.82	1.23
<i>N. sphaericha</i>	-	-	-	-	-	-	-	0.06	0.13	-	-	0.13	0.20	-	0.20	0.34	0.61	0.27	0.34	0.55	0.75
<i>Achlya</i> sp.	-	-	-	-	-	-	-	-	-	-	-	0.06	0.13	-	0.20	0.27	0.34	0.06	0.20	0.48	0.68
<i>Papulaspora immerse</i>	-	-	-	-	-	0.06	0.20	0.34	0.48	0.13	0.27	0.55	0.75	0.20	0.48	0.61	1.031	0.27	0.55	0.82	1.23
<i>Aphanomyces</i> sp.	-	-	-	-	-	-	-	-	0.06	-	-	0.06	0.13	-	0.06	0.20	0.34	0.13	0.41	0.61	0.89
Total	-	0.34	0.61	0.61	0.75	0.61	0.95	2.37	4.54	1.2	2.78	5.03	7.19	2.71	5.37	7.94	11.50	4.32	8.44	13.13	17.98

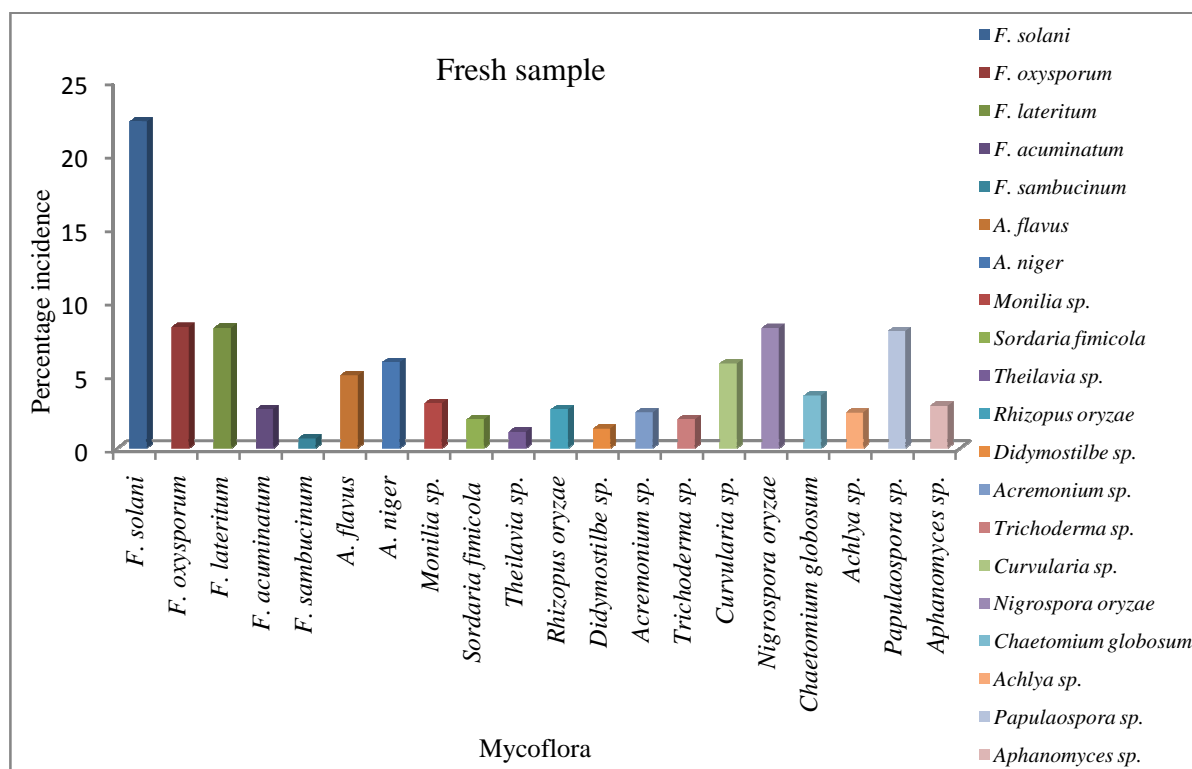
Table2: Percentage incidence of fungal isolated from the root of *Aegle marmelos* (Market sample) stored at various relative humidity

Mycoflora	con	30%				50%				75%				96%				100%			
		15	30	45	60	15	30	45	60	15	30	45	60	15	30	45	60	15	30	45	60
<i>Aspergillus niger</i>	0.42	-	-	0.42	0.71	0.14	0.56	0.85	1.28	0.42	1.28	2.13	3.98	0.56	0.71	1.56	4.55	0.71	0.99	2.56	4.98
<i>Aspergillus flavus</i>	0.14	-	-	-	0.28	-	0.42	0.56	0.56	-	0.28	0.71	0.99	0.14	0.42	0.99	1.28	0.28	0.99	1.56	2.13
<i>Scytallidium sp.</i>	-	-	0.14	0.28	0.42	0.14	0.14	0.28	0.56	0.14	0.41	0.71	0.85	0.24	0.42	0.99	1.28	0.28	0.41	1.28	2.13
<i>Papulaspora immerse</i>	0.14	0.14	0.14	0.14	0.28	-	0.14	0.28	0.42	0.14	0.56	0.71	0.85	0.14	0.56	0.71	0.99	0.28	0.56	2.13	1.28
<i>Rhizopus oryzae</i>	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.28	0.28	0.14	0.28	0.41	0.56	0.14	0.28	0.71	0.85	0.14	0.71	0.99	1.56
<i>Monilia sitophila</i>	0.14	-	-	0.14	-	-	-	0.14	0.28	-	0.14	0.14	0.42	-	0.14	0.56	0.99	0.14	0.71	0.99	1.28
<i>Nigrospora oryzae</i>	-	-	-	-	-	-	-	-	-	-	-	-	0.14	-	0.28	0.42	0.56	0.14	0.71	1.13	1.56
<i>Sporothrix sp.</i>	0.14	-	-	-	-	-	-	0.14	0.14	-	0.14	0.14	0.14	-	0.14	0.28	0.41	0.14	0.41	0.71	1.13
<i>Aspergillus parasiticus</i>	-	-	-	-	-	-	-	-	-	-	-	-	0.14	-	-	0.28	0.71	0.14	0.85	0.85	0.99
<i>Aspergillus fumigatus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.14	0.28	0.42	0.14	0.56	0.71	0.85
<i>Curvularia lunata</i>	-	-	-	-	-	-	-	-	-	-	-	-	0.14	-	-	0.14	0.28	0.14	0.56	0.71	0.99
<i>Cunningamella elegans</i>	-	-	-	-	-	-	-	-	-	-	-	-	0.14	-	-	0.14	0.28	0.14	0.41	0.41	0.71
<i>Aspergillus sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.14	0.28	0.42
<i>Nigrospora sphaericha</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.14	-	0.14	0.14	0.41	
<i>Aspergillus tamarii</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.14	0.56
Total	1.20	0.28	0.42	1.12	1.83	0.42	1.4	2.53	3.52	0.84	3.09	4.95	8.35	1.22	3.09	7.06	12.74	2.67	8.15	14.59	18.15

Table3: Deterioration of total alkaloids content (mg/100mg) in root of *Aegle marmelos* (Fresh and market samples) at different relative humidities

Incubation days	Control	30%	50%	75%	96%	100%
1 day	16.56±0.32	16.56±0.32	16.56±0.32	16.56±0.32	16.56±0.32	16.56±0.32
15days	16.56±0.32 ^c	16.56±0.25 ^c	16.56±0.15 ^{bc}	16.56±0.23 ^b	16.56±0.25 ^a	16.56±0.26 ^a
30days	16.56±0.32 ^c	16.56±0.32 ^c	16.53±0.20 ^b	16.43±0.20 ^b	16.36±0.20 ^a	16.30±0.17 ^a
45 days	16.56±0.32 ^d	16.56±0.32 ^c	16.02±0.15 ^b	16.6±0.15 ^a	16.02±0.068 ^a	15.98±0.026 ^a
60 days	16.56±0.26 ^d	16.26±0.25 ^c	16.03±0.057 ^b	15.97±0.025 ^{ab}	15.96±0.01 ^a	15.93±0.025 ^a
75 days	16.56±0.25 ^d	16.06±0.11 ^c	15.94±0.041 ^b	15.94±0.015 ^{ab}	15.87±0.020 ^a	15.84±0.049 ^a
90 days	16.56±0.3 ^d	15.96±0.06 ^c	15.91±0.017 ^{bc}	15.85±0.037 ^a	15.84±0.037 ^a	15.80±0.015 ^a

Incubation days	Control	30%	50%	75%	96%	100%
1 day	16.62±0.01	16.62±0.01	16.62±0.01	16.62±0.01	16.62±0.01	16.62±0.01
15days	16.62±0.11 ^b	16.61±0.11 ^b	16.61±0.31 ^a	16.62±0.23 ^a	16.59±0.12 ^a	16.58±0.26 ^a
30days	16.61±0.11 ^b	16.60±0.12 ^b	16.60±0.11 ^b	16.59±0.20 ^b	16.40±0.20 ^a	16.30±0.15 ^d
45 days	16.60±0.10 ^c	16.55±0.06 ^b	16.47±0.068 ^a	16.41±0.01 ^a	16.27±0.081 ^a	16.20±0.081 ^a
60 days	16.60±0.073 ^c	16.37±0.051 ^{dc}	16.10±0.17 ^{bc}	15.95±0.023 ^{ab}	15.80±0.18 ^{ab}	15.76±0.23 ^a
75 days	16.58±0.063 ^d	15.97±0.025 ^c	15.89±0.017 ^b	15.82±0.02 ^{ab}	15.78±0.032 ^a	15.76±0.052 ^a
90 days	16.57±0.095 ^d	15.92±0.045 ^c	15.83±0.051 ^{bc}	15.72±0.048 ^{ab}	15.71±0.037 ^b	15.66±0.057 ^a

Graph1. Percentage incidence of mycoflora associated with the root of *Aegle marmelos* (Fresh samples)

Market sample of this drug stored at same relative humidity and deterioration of total glycosides observed, at the first day 9.26% recorded for total amount of glycosides, more deterioration showed in market sample as compared to fresh sample. After 90 days of incubation period amount of total glycosides showed more deterioration under all tested RH, 8.73 (30 % RH), 8.36 (50 % RH), 8.35 (75 % RH), 8.26 (96 % RH) and 8.17 % (100% RH) (Table4).

Data analysis of variance indicates that deterioration of total alkaloids and glycosides contents are under influence of incubation days and relative humidity and they are significant at 5% level significance (P value <0.05).

The principal factors on fungal growth and deterioration are moisture, temperature, atmosphere, aeration, PH, condition of the storage and incubation period of herbal plants. All of these factors interact as deterioration progresses, but moisture and temperature are probably most important. Christensen (1957) grouped fungi into two categories: field fungi and storage fungi. This division is not taxonomically valid but is based primarily upon moisture requirements. Field fungi attack developing and mature tissue of herbal drugs; storage fungi are usually encountered when organs of medicinal plants are stored after harvest. The major field fungi are species of *Alternaria*, *Cladosporium*, *Fusarium* and *Helminthosporium*; although species of *Curvularia*, *Stemphylium*,

Epicoccum and *Nigrospora* are included in this group. Storage fungi are predominantly species of *Aspergillus* and *Penicillium*. Species of *Aspergillus* are not always well defined and are sometimes referred to as "groups." The major storage fungi consist of five or six groups of *Aspergillus*, plus several species of *Penicillium* which are common until deterioration is well advanced (Christensen and Kaufmann 1974). Certain other species of *Penicillium* are considered field fungi (Mislivec and Tuite 1970). For the most part, the above categories are accurate; however, exceptions exist, *A. flavus* can invade in the field and *Fusarium* can continue to decay of plant tissues in storage if the moisture is high enough (Caldwell and Tuite, 1974). There is a correlation between the growth and incidence of fungi with high relative humidity and prolonged incubation days. The predominant fungi associated with drug roots *Fusarium* and *Aspergillus* species which both are toxigenic fungi and under suitable condition can invade to tissue plants and produce toxin, which consumption of these contaminated materials by human leads to several physiological disorders and even death. Therefore, there is an urgent need to prevent the entrance of such contaminated crude drug samples into commercial herbal drugs.

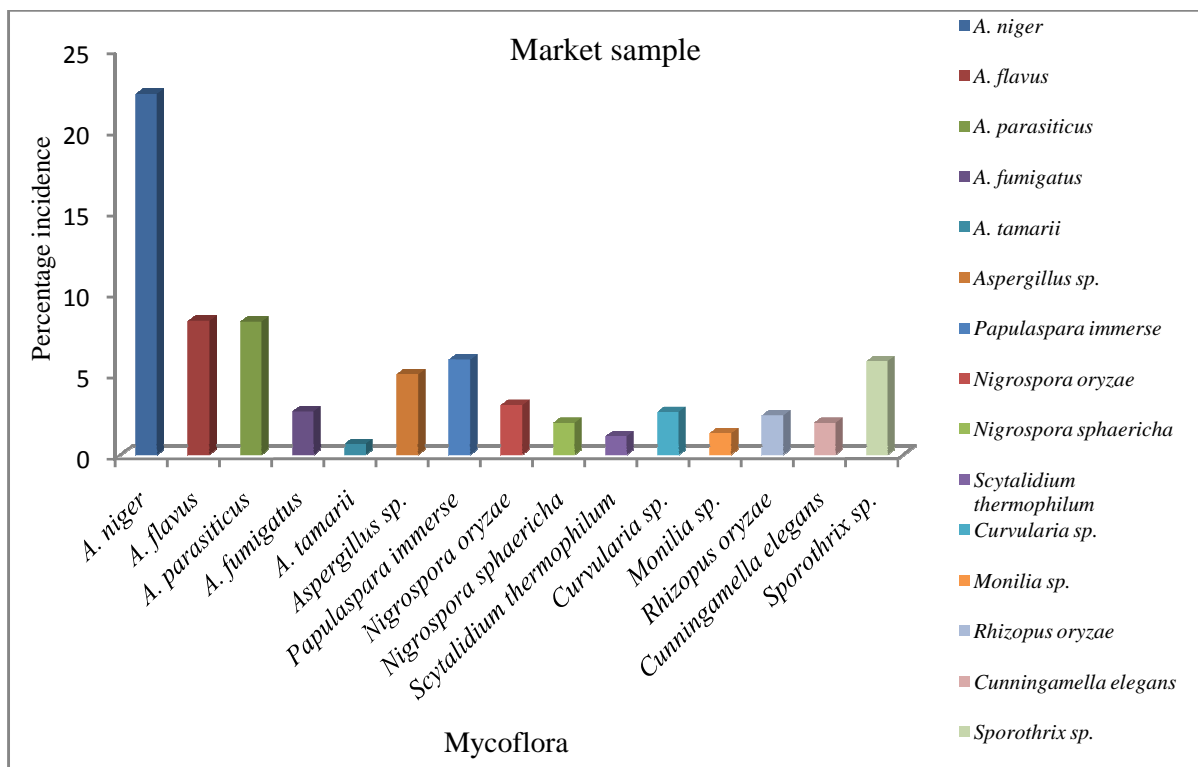
Table 4: Deterioration of total glycosides content (mg/100mg) in root of *Aegle marmelos* (Fresh and market samples) at different relative humidities

Incubation days	Control	30%	50%	75%	96%	100%
1 day	9.53±0.032	9.53±0.032	9.53±0.032	9.53±0.032	9.53±0.032	9.53±0.032
15days	9.53±0.037 ^d	9.48±0.052 ^d	9.41±0.01 ^c	9.38±0.015 ^c	9.3±0.043 ^b	9.53±0.032 ^a
30days	9.53±0.030 ^d	9.4±0.068 ^d	9.31±0.01 ^c	9.27±0.051 ^c	9.15±0.015 ^b	9.09±0.049 ^a
45 days	9.53±0.028 ^d	9.3±0.095 ^d	9.18±0.025 ^c	9.09±0.095 ^c	8.91±0.055 ^b	8.75±0.10 ^a
60 days	9.53±0.096 ^d	9.18±0.064 ^d	9.001±0.064 ^c	8.81±0.15 ^b	8.78±0.047 ^b	8.61±0.085 ^a
75 days	9.53±0.045 ^d	8.89±0.086 ^d	8.81±0.032 ^c	8.73±0.032 ^{bc}	8.73±0.02 ^b	8.63±0.047 ^a
90 days	9.53±0.032 ^d	8.74±0.045 ^d	8.32±0.11 ^c	8.41±0.072 ^{bc}	8.31±0.026 ^b	8.2±0.032 ^a

Incubation days	Control	30%	50%	75%	96%	100%
1 day	9.26±0.13	9.26±0.13	9.16±0.23	9.16±0.23	9.16±0.23	9.26±0.013
15days	9.26±0.12 ^d	9.26±0.12 ^c	9.26±0.13 ^b	9.26±0.015 ^a	9.26±0.43 ^a	9.25±0.13 ^a
30days	9.26±0.12 ^d	9.26±0.068 ^c	9.26±0.01 ^b	9.24±0.14 ^a	9.23±0.015 ^b	9.21±0.12 ^a
45 days	9.25±0.13 ^d	9.25±0.12 ^c	9.25±0.13 ^c	9.22±0.12 ^b	9.14±0.11 ^b	8.92±0.10 ^a
60 days	9.24±0.13 ^d	9.02±0.062 ^c	8.96±0.058 ^c	8.76±0.056 ^b	8.66±0.058 ^b	8.55±0.061 ^a
75 days	9.24±0.045 ^d	8.78±0.13 ^c	8.84±0.14 ^c	8.63±0.055 ^b	8.53±0.079 ^b	8.28±0.09 ^a
90 days	9.23±0.032 ^d	8.73±0.062 ^c	8.36±0.15 ^b	8.35±0.04 ^{ab}	8.26±0.056 ^b	8.17±0.025 ^a

Data are the mean of three replicates ± standard deviation. P- Value denoted the significance of differences between the mean by univariate comparison statistics. The value followed by different letters differ significantly by Duncan's multiple rang test at P=Sig= 0.05

Grap2. Percentage incidence of mycoflora associated with the root of *Aegle marmelos* (market samples)



Deterioration of alkaloids and glycosides amounts under different relative humidities due to spoilage of fungi which might be due to its degradation into simpler forms of chemical constituents by enzymes which produce by fungi and their utilization as a source of energy for their growth. This was in accordance with the result of earlier workers (Deokule and Kabnoorkar 2003, Dutta and Roy 1987, Kabnoorkar and Deokule 2009).

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