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Inhibition of production of aflatoxin by bioactive compound 2H-furo[2,3-H]-1-benzopyran-2-one isolated from the seeds of *Psoralea corylifolia* L.

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Abstract

Forty maize seeds samples collected from different places of Karnataka were subjected for Standard Blotter Method and the seeds borne pathogens were isolated . Among different pathogens isolated, Aflatoxin producing fungi *Aspergillus flavus* were identified. Aqueous extract of *Psoralea corylifolia* were prepared at different concentrations and subjected for antifungal activity by poisoned food technique for eight *Aspergillus* species selected. Among eight *Aspergillus* species tested, *Aspergillus* species No. 6 recorded 93.4% inhibition at 50% concentration. Bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one, isolated form the seeds of *P. corylifolia* were teased for antifungal activity against different strains of *A. flavus* at 250, 500, 750 and1000ppm concentration respectively and the maximum inhibition was observed in 1000ppm concentration and subjected for the isolation of aflatoxin compared with standard aflatoxin. It was observed that, Aflatoxin B1(R_f 0.56) isolated from *A.flavus* strain no.6 was completely inhibited by bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one, confirming the inhibition of Aflatoxin from *P. corylifolia* seeds.

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Introduction

Over 300 mycotoxins have been described, but much of the attention has been concentrated on aflatoxins and trichothecens and on secondary groups such as citerinin, cyclopiazonic acid, ochratoxins, patulin, sterigmatocystin and zearalenone (Agarwal and Sinclair, 1997). In recent times, incidence of mycotoxins and mycotoxigenic fungi in food, feeds and fodders has been reported from different parts of the world (Rafiuddin *et al.*, 1999). World food production has grown faster than the population. Inspite of the progress achieved in increasing food supply, a large population of the world especially in the developing countries are undernourished. Although developing countries are claiming that they are self sufficient in food supply, the quality of the commodities and the health security of those who consume them are at risk. One of the important factors contributing to the non availability of enough food to the growing population is the colonization of moulds in food grains and commodities and health hazardous mycotoxin production.

Mycotoxins are fungal secondary metabolites formed by consecutive series of enzyme catalyses reactions. The main genera of fungi associated with mycotoxins include species of *Aspergillus, Penicillium, Fusarium, Claviceps, Alternaria, Pithomyces, Stachybotrys, Phoma* and *Diplodia* (Bohra and Purohit, 2003). The toxins can accumulate in maturing corn, cereals, soybeans, sorghum, peanuts and other food and feed crops in the field and in grains during transportation and storage (Jacobsen *et al.*, 2000). Human suffering from mycotoxicoses includes ergot poisoning associated with ingestion of rye flour contaminated with ergot, cardiac beriberi associated with *Penicillium* moulds in rice and alimentary toxin aleukia associated with *Fusarium* moulds on over wintered wheat, millet and barley. Several mycotoxins have been linked to increased incidence of cancer in human beings. These include aflatoxins, sterigmatocystin, zearalenone, patulin, ochratoxin and fumonisin (Jacobsen *et al.*, 2000). There are many reports on the effect of mycotoxins on human beings. The alimentary toxic aleukia (ATA) observed in Russia during the 19th century, which was widespread during world war II and the post war years until 1947, is caused by the consumption of mouldy seeds. An outbreak of Turkey X disease in Great Britain in 1960, was traced to contaminated peanut meal from Brazil. Aftatoxin was indicated as the cause of the death for more than 100,000 young turkeys and 20,000 ducklings, pheasants and partridge pouts (Jacobsen *et al.*, 2000).

Mycotoxicosis is systemic and serious with tissue degeneration, immunosupression, carcinogenesis and genotoxicity, all recorded in both livestock and humans (Mabbett, 2003). Cereals are highly susceptible to fungal growth, in the field, during processing and during storage. Small grains (wheat, sorghum, oats, rye, barley and rice) unless abused in storage appears to be less susceptible to mycotoxins formation than the larger grain such as maize and groundnut (Jacobsen *et al.*, 2000; Bohra and Purohit, 2003). Mycotoxins cause large economic losses to crop producers and food processors. “It is estimated that 10-30% of the harvested grains are lost due to the mould infection”.

FAO estimates that about 25% of the world's food crops are affected by mycotoxins (Cirillo *et al.*, 2003). Approximately 20-30% of food becomes unsafe before it reaches the consumers. Human poisoning by fungal metabolites caused due to consumption of contaminated food stuff has been a matter of serious concern in different parts of the world including India (Bohra and Purohit, 2003).

Aflatoxins are one of the highly toxic secondary metabolites derived from polyketides produced by fungal species such as *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* (Payne and Brown, 1998). The major aflatoxins are B1, B2, G1, and G2, which can poison the body through respiratory, mucous or cutaneous routes, resulting in over activation of the inflammatory response (Romani, 2004). *A flatoxin* B1, B2, G1 and G2 are the most common mycotoxins identified and severely infected in cereals .Theses toxins production are enhanced during food

production, harvest, storage and processing. Consumption of mycotoxins may cause decreased resistance to infectious diseases and impaired immunity (Yasemin and Gamze, 2004). This toxins when exposed to human for long period, it results in severe human health issues such as retardation, cirrhosis, hepatocellular carcinoma and other complications. Aflatoxins are poisonous mycotoxins produced by moulds in soil, decaying vegetation, hay, and grains. They affect cereals, oilseeds, spices, and tree nuts. Contaminated feed can also contain aflatoxins in animal milk. Large doses can cause acute poisoning, liver damage, and genotoxicity, damaging DNA and potentially causing cancer in animals and humans (WHO, 2023). Aflatoxin can have a negative impact on the physiological status of humans and animals by causing developmental abnormalities in embryos under long-term exposure (Alameri *et al*., 2023). *Aspergillus flavus*, a fungus that causes an ear mold in maize since 1920, was initially overlooked until the 1960s when it was found to produce aflatoxin, a factor linked to Turkey X disease. This discovery in the Southern and Midwestern US in the 1970s highlighted the importance of pre-harvest contamination (Payne,1998; Widstrom, 1996). In the present investigation, bioactive compound isolated from the seeds of *Psoralea corylifolia* belongs to family Fabaceae were evaluated for the management of seed borne fungi and *Aspergillus flavus* species and its aflatoxin production inhibition in *in vitro* condition.

Materials and methods

Plant material

Shade dried, healthy seeds of *P. corylifolia* were collected from seed market, Mysore. The seeds were washed thoroughly 2-3 times with running tap water and once with sterile distilled water, air dried at room temperature on a sterile blotter. After completion of drying seeds were used for preparation of aqueous extract.

Preparation of aqueous extract

One hundred grams of the thoroughly washed and air dried healthy seeds of *P. corylifolia* were macerated with 100 ml of sterile distilled water in a waring blender (Waring International, New Hartford, CT, USA) for five min. The macerate was filtered through double-layered muslin cloth, and then centrifuged at 4000g for 30 minutes. The supernatant was filtered through Whatman No.1 filter paper and sterilized at 120°C for 10 minutes, which served as 100% aqueous mother extract. The extract was preserved aseptically in a sterile brown bottle at 5° C until further use (Pinto *et al*., 1998).

Collection of seed samples

Naturally infected forty seed samples of five varieties of maize (*Zea mays* L.) Were collected from different places of Karnataka. The seeds were surface sterilized with 0.1% sodium hypochloride and rinsed with distilled water for four to five times, air dried and subjected for standard blotter method. The list of maize seed samples collected their variety and the place of collection is presented in Table.1.

Isolation and Identification of Biodeterioration Causing Fungi in Maize

Standard blotter method was employed for isolation of seed borne biodeterioration causing fungi. Three layers of blotters equivalent to the size of the Petridis were soaked in distilled water, the surplus water is drained from the blotters and placed in the lower lid of the Petridis. Eight verities of four hundred seeds of each of the samples were placed on the blotters at the rate of ten seeds per plate. These plates were incubated for seven days at 22±2°C under alternating cycles of 12/12 hours of NUV light and darkness. After the period of incubation the seeds were observed under stereobinocular microscope and the *Aspergillus flavus* species associated with these seeds were identified based on their growth habit, mycelial structure and spore morphology using standard manuals. The diversity of the fungal species were recorded and the percentage of infection of each of the fungi were determined (ISTA, 1999).

All the fungi associated with the seeds were isolated on Yeast Extract Sucrose (YES) agar medium and their pure cultures were maintained on specific media. The fungi were sub-cultured periodically.

Test fungi

Eight isolates of *Aspergillus flavus* isolated from maize seeds were used as test fungi for antifungal activity assay .All the fungi were identified using standard manual and pure cultures were preserved in lower temperature for further use (Verma and Dohroo, 2003).

Preparation of inoculum

Yeast Extract Sucrose (YES) broth was prepared and pure culture of *A. flavus* strain 6 was inoculated aseptically and incubated for seven to ten days.

Antifungal activity assay by poisoned food technique

Yeast Extract Sucrose (YES) medium with different concentrations of the aqueous extract of seeds of *P. corylifolia* viz., 10, 20, 30, 40, and 50% were prepared and poured into sterile petriplates, and allowed to cool and solidify. Five mm mycelial discs of seven-day-old cultures of species of all *Aspergillus* isolates were placed at the centre of the Petri plates and incubated at 25 ±1° C for seven days. The Yeast Extract Sucrose medium without the aqueous extract but with the same concentration of sterile distilled water served as a control. The colony diameter was measured in mm. For each treatment three replicates were maintained. The percentage inhibition of mycelial growth, if any, was determined by the formula PI = C-T/ C x 100, where C = diameter of control colony and T = diameter of treated colony (Romani, 2004).The minimal inhibitory concentration (MIC) for each of the test fungi was determined following the procedure of 10. The data was subjected to statistical analysis by ANOVA and Tukey's HSD.

Chemical fungicides

Two chemical fungicides viz., Bavistin, and Thiram were evaluated for antifungal activity by poisoned food technique for comparison.

Isolation of the Bioactive compound from the seeds of P. corylifolia

Twenty five grams of the powdered seeds of P. corylifolia were extracted from petroleum ether and methanol mixture in the ratio 9:1(v/v) by refluxing for eight hours at 50- 60°C in a Soxhlet apparatus.

The excess of solvent was removed by distillation under reduced pressure. The concentrated extract was cooled for 48 hrs at 5°C to obtain pure compound as crystals. The crystals was dissolved in chloroform and eluted by silica gel (6×120 mesh size) column chromatography (2×40 cms) using n-hexane: chloroform (9:1)(v/v). The purity of the bioactive compound was confirmed by TLC and R_f value was determined.

Structural elucidation of the Bioactive compound

IR (Infra red) analysis

IR spectra were recorded in KBr pellete on a Perkin-Elmer FTIR 1650 spectrometer (Al-Fatimi *et al.*, 2006).

^{1}H- NMR data analysis

The ^{1}H- NMR spectrum of the bioactive compound, 2H-Furo [2,3-H]-1-benzopyran-2-one was recorded on a Bruker AM 400 F (400 MHZ) NMR spectrometer using $CDCI_3$ as a solvent and TMS as internal standard. All chemical shift values were expressed in δ scale as s= singlet, d= doublet, t= triplet, m= multiplet (Al-Fatimi *et al.*, 2006).

^{13}C NMR analysis

The ^{13}C-NMR spectra were obtained on a Burker spectrometer AM 400(400 MHZ) with the solvent signal as internal reference (Al-Fatimi *et al.*, 2006).

Gas Chromatography- Mass Spectroscopy (GC- MS) analysis

Mass spectrum of compound was recorded on MS data review active chromatogram with range 40-600M/Z [Yanez *et al.*, 2005]. The other characterizations of the data of the compound were also determined.

Treatment of different concentration of bioactive compound isolated from the seeds of P.corylifolia on Aspergillus flavus strain 6

The YES broth was prepared and subjected for autoclaving for 15 minutes and after autoclaving, the

media was allowed to cool. 100ml of YES broth was transferred to each five sterilized and autoclaved 200ml conical flask. To each five set of conical flask, 50 microliter of *A. flavus* strain 6 fungal spore suspension which consists of 10^5 spores /ml concentration was added. This is considered as one set 1 conical flask. The same was repeated to maintain five sets of conical flask as set 2, set 3, set 4 and set 5. To set 1 conical flasks, 250ppm of bioactive compound isolated from seeds of *P. corylifolia* was added, similarly 500ppm was added to set 2, 750ppm was added to set 3 and 1000ppm was added to set 4. To the set 5, bioactive compound was not added which serves as control. All the conical flasks was incubated for 7 days. After 7 days of incubation, all the culture conical flasks was filtered to remove any mycelial growth of *A. flavus* and subjected for estimation of aflatoxin production. After the incubation period, the contents of each of the flask were filtered through a pre-weighted Whattman No.1 filter paper. The filter papers with the mycelial mats were dried in an oven at 100°C until constant weights were obtained. The mycelial dry weight was determined by substracting the weight of the filter paper from the total weight (Venturini *et al.*, 2022).

Extraction of aflatoxin

100ml of *A.flavus* strain 6 broth which was incubated by above method was taken in one litre blender jar, add 500ml of methanol/water(55:45 v/v), 200ml of hexane and 4 grams sodium chloride. Blend at high speed for approximately 1 minute . the mixture was allowed to settle for 30 minutes for separation. 25 ml of the aqueous methanol layer was taken in a 125 ml separating funnel and extracted with 25ml chloroform. Lower chloroform layer was drained off through anhydrous sodium sulphate bed into 100ml beaker. Dry I on a water bath and transfer into a vial by dissolving in 1 ml of chloroform (Kulwant *et al.*, 1991).

Preparation of activated TLC plate

Prepare TLC plates of silica gel G (8-10 g with about 20 ml of water on a plate of 20 x 20 cm size) using an applicator (0.40 mm thickness). Air-dry the plates overnight. Activate the plates in oven at 110 ± 1°C for 1 h. Dilute the dried extract to a fixed volume (e.g. 200 µl) with benzene: acetonitrile mixture (98:2, v/v). Apply 5 or 10 µl of the diluted extract on the marked TLC plates (3 cm above the bottom edge of the plate) along with standard aflatoxin solution representing 2.5, 5.0, 7.5 and 10 µg concentrations. Run the plates to about 15 cm height in a solvent system (chloroform: acetone, 90:10 v/v). Remove the plates and air dry. Observe the plates under UV light at 360 nm in a UV chromatography view cabinet. Compare the intensities of the fluorescent of the spots produced by the sample with those produced by the standard for quantifying the aflatoxin content in the sample.

$$\text{Aflatoxin B content (µg/kg)} = \frac{C \times V \times V}{M \times V}$$

Where,

C - Concentration of aflatoxin B1 from calibration curved solution (µg/ml)

V1 - Final volume of extract

V2- Volume of extract applied on the TLC plate.

V3- Volume of standard B solution applied on the TLC plate whose

fluorescence intensity is similar to that of sample (µg)

M - Weight of the sample (Kulwant *et al.*, 1991).

Determination of aflatoxin by TLC

5 micro litre of *A. flavus* strain 6 extract was spotted on developed TLC plates. For comparing and confirming Aflatoxin B1, standard Aflatoxin B1 was also spotted on the same TLC plates. The elutent used for TLC is toluene/ethylacetate/90% formic acid(TEF)(60:30:10 v/v) until the solvent front moves upto 10 cms. After running the TLC plates with elutents, the plates were dried. The dried plates were observed under long wave(365nm) UV light and shortwave (254nm) UV light and recorded the spots generated. Blue fluorescence observed under long wave UV light at R_f 0.56 confirm the presence of Aflatoxin B1 (Kulwant *et al.*, 1991).

Conformation of Aflatoxin B1

5 and 10 micro litre of sample extract supposed to

contain aflatoxin from above extraction was spotted . 2 microliter of trifluroacetic acid (TFA) was superimposed on the spot supposed to contain aflatoxin. And the loaded TLC plates was eluted by toluene/ethylacetate/90% formic acid (TEF) (60:30:10 v/v) until the solvent front moves upto 10 cms. Observe the plates under long wave UV light (Kulwant *et al.*, 1991).

Statistical analysis

The data were subjected to Tukey's HSD analysis. Data on percentages were transformed to arcsine and analysis of variance (Anova) was carried out with transformed values. The means were compared for significance using Tukey's HSD (P=0.05).

Results

Isolation and identification of biodeterioration causing fungi in maize

Percent incidence and relative preponderance of seed borne fungal pathogens in maize

The seed samples collected from different sources provided enough scope for the inclusion of varied cultivars from different localities for the inspection and open analysis. It was observed that irrespective of the places and sources concerned, maize seeds carried several diverse types of fungi (Table 2).

Thirty seven fungal species belonging to eighteen genera were detected from forty seed samples. The fungal species included both field and storage fungi.

Table 1. List of maize seed samples collected.

Sample No.	Place of collection	Variety	Source
1.	Mysore	Deccan	Farmer storage
2.	Mandya	Deccan	Farmer storage
3.	Bannur	Deccan	Farmers field
4.	Mysore	Deccan	Market sample
5.	Bangalore	Deccan	Market sample
6.	Mandya	Deccan	Market sample
7.	K.R.Nagar	Deccan	Market sample
8.	H.D. Kote	Deccan	Market sample
9.	Hunsur	Deccan	Retail shop
10.	Mysore	Deccan	Retail shop
11.	Mysore	Deccan	APMC yard
12.	Kolar	Deccan	APMC yard
13.	Shimoga	Deccan	APMC yard
14.	Ramnagaram	Ganga-1	APMC yard
15.	Hunsur	Ganga-1	APMC yard
16.	Bangalore	Ganga-1	APMC yard
17.	Mysore	Ganga-1	Market sample
18.	Hassan	Ganga-1	Market sample
19.	Chikmangalore	Ganga-1	Market sample
20.	Mysore	Ganga-101	Market sample
21.	Chamarajanagar	Ganga-101	Market sample
22.	Kollegal	Ganga-101	Farmer storage
23.	T.Narasipura	Ganga-101	Farmer storage
24.	Srirangapatana	Ganga-101	Farmer storage
25.	Chennapatana	Ganga-101	Farmer storage
26.	Maddur	Ganga-101	Farmer storage
27.	Haveri	Ganga-101	Farmer storage
28.	Hubli	Ganga-101	Farmer storage
29.	Dharwad	Himalaya-123	Farmer storage
30.	Mysore	Himalaya-123	Retail shop
31.	Gonikoppal	Himalaya-123	Retail shop
32.	Sakaleshpura	Himalaya-123	Retail shop
33.	Hassan	Himalaya-123	Retail shop
34.	Holenarisipura	Himalaya-123	Retail shop
35.	K.R.Nagar	Himalaya-123	Retail shop
36.	Mysore	Ranjit	Farmers field
37.	Kollegal	Ranjit	Farmers field
38.	H.D.Kote	Ranjit	Farmers field
39.	Hunsur	Ranjit	Farmers field
40.	Mandya	Ranjit	Farmers field

Some of the important fungal species which were observed frequently in many seeds samples with higher percentage of infection were species of *Aspergillus* viz., *A. fumigatus, A. candidus, A.*

columnaris, A. flavus, A. flavus oryzae, A. nidulans, A. ochraceous, A. tamarii, A. terreus and *A. flavipes, Fusarium* viz., *F. graminearum F. moniliforme F. oxysporum, F. solani, F. equiseti, F. lateritium, F. semitectum* and *F. proliferatum* and *Penicillium viz., P. chrysogenum* and *P. notatum* (Table 2). More than 55% of seed samples were associated with diverse species of field fungi and about 45% of the seed samples were heavily infected with biodeterioration causing storage fungi, which included species of *Aspergillus* and *Penicillium*. The relative preponderance of the fungi that occurred frequently on different seed samples has been presented in Table 2. About 20 fungal species were frequently observed in more than 50% of the seed samples at varying levels of infection. Observations clearly indicated that species of *Aspergillus, Fusarium, Penicillium* and *Rhizopus* are frequently associated with the seed samples collected from this region.

Table 2. Relative preponderance of the fungi of maize seeds.

Sl. No	Fungi	% Incidence				No of seeds samples on which the fungus was encountered
		0-10	11-30	31-50	51-100	
1.	A. fumigatus	13	8	2	-	23
2.	*A. candidus*	12	10	1	1	24
3.	*A. columnaris*	17	8	-	-	25
4.	*A. flavipes*	4	11	2	3	20
5.	*A. flavus*	2	15	13	10	40
6.	*A. flavus oryzae*	11	9	4	-	24
7.	*A. nidulans*	19	4	2	-	25
8.	A. niger	6	15	10	4	35
9.	*A. ochraceous*	16	5	1	1	23
10.	*A. tamarii*	14	7	3	-	24
11.	*A. terreus*	17	16	2	1	36
12.	*Alternaria alternata*	12	2	-	-	14
13.	*Botridiplodia*	4	-	-	-	4
14.	*Chaetomium globosum*	10	-	-	-	10
15.	*Cladosporium cladosporides*	15	1	-	-	16
16.	*Curvularia lunata*	10	8	-	-	18
17.	*D.longistrata*	7	8	2	-	17
18.	*Drechslera halodes*	4	8	-	1	13
19.	*F. graminearum*	23	3	-	-	26
20.	*F. moniliforme*	6	12	11	4	33
21.	*F. oxysporum*	7	13	3	-	23
22.	*F. solani*	12	11	2	-	25
23.	*F.equiseti*	9	8	2	2	21
24.	*F.lateritium*	11	8	4	1	24
25.	*F.semitectum*	20	7	-	1	28
26.	*F.proliferatum*	9	8	2	2	21
27.	*Macrophomina*	11	3	-	-	14
28.	*Memnoniella*	2	3	-	-	5
29.	*Nigrospora*	5	6	-	-	11
30.	*Penicillum chrysogenum.*	10	14	4	1	29
31.	*Penicillum* sp.	16	14	-	-	30
32.	*Phoma* sp.	6	-	-	-	6
33.	*Phomopsis*	4	-	-	-	4
34.	*Pithomyces*	5	-	-	-	5
35.	*Rhizopus*	9	10	-	-	19
36.	*Sclerotium rolfsii*	5	-	-	-	5
37.	*Trichoderma viride*	6	11	1	-	18

Antifungal activity assay by poisoned food technique

Among the eight *A. flavus* strains selected, maximum inhibition was observed in *A. flavus* strain 6 and recorded 93.4% inhibition at 50% concentration of aqueous extract. 81.3% inhibition was recorded at 40% concentration, 68.9% inhibition at 30% concentration, 39.2% inhibition at 20% concentration and 19.2% inhibition at 10% concentration respectively. *A.flavus* strain 6 was followed by *A. flavus* strain 3 and recorded 90.2% inhibition at 50% concentration, 89.5% inhibition in *A.flavus* strain 4 and 87.7% inhibition in *A. flavus*

strain 3 respectively at 50% concentration. Least inhibition was observed in *A. flavus* strain 1 and recorded 52.1% inhibition at 50% concentration. The percentage of inhibition goes on increasing with increasing the concentration. Compared to synthetic fungicides, Bavistin and Thiram at 2.0% recommended concentration, both fungicides recorded 100% inhibition against all the test fungi tested (Table 3).

Table 3. Antifungal activity of aqueous extract of seeds of *P.corylifolia* L. against seed borne fungi of maize.

Fungi	Mycelial Growth Inhibition(%)						
	Concentration of Aqueous Extract					Bavistin	Thiram
	10%	20%	30%	40%	50%	2%	2%
A.flavus strain 1	10.0^{a} ±0.1	18.9^{b} ±0.1	28.3^{c} ±0.1	36.1^{d} ±0.1	52.1^{e} ±0.1	100.0^{f} ±0.0	100.0^{f} ±0.0
A.flavus strain 2	16.2^{a} ±0.0	29.3^{b} ±0.1	42.7^{c} ±0.2	65.2^{d} ±0.1	87.7^{e} ±0.0	100.0^{f} ±0.0	100.0^{f} ±0.0
A.flavus strain 3	18.1^{a} ±0.1	38.0^{b} ±0.0	51.3^{c} ±0.0	72.9^{d} ±0.0	90.2^{e} ±0.1	100.0^{f} ±0.1	100.0^{f} ±0.1
A.flavus strain4	16.2^{a} ±0.0	28.9^{b} ±0.0	45.9^{c} ±0.1	68.9^{d} ±0.0	89.5^{e} ±0.2	100.0^{f} ±0.0	100.0^{f} ±0.2
A.flavus strain 5	10.0^{a} ±0.2	23.9^{b} ±0.0	43.9^{c} ±0.0	61.1^{d} ±0.0	78.0^{e} ±0.1	100.0^{f} ±0.2	100.0^{f} ±0.1
A.flavus strain 6	19.2^{a} ±0.0	39.2^{b} ±0.0	68.9^{c} ±0.0	81.3^{d} ±0.0	93.4^{e} ±0.0	100.0^{f} ±0.0	100.0^{f} ±0.0
A.flavus strain 7	12.4^{a} ±0.0	21.0^{b} ±0.0	38.4^{c} ±0.0	56.8^{d} ±0.1	73.2^{e} ±0.1	100.0^{f} ±0.0	100.0^{f} ±0.0
A.flavus strain 8	11.2^{a} ±0.0	23.9^{b} ±0.0	43.1^{c} ±0.0	60.1^{d} ±0.0	78.9^{e} ±0.1	100.0^{f} ±0.1	100.0^{f} ±0.1

*Values are the mean of three replicates, ±standard error

*The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey"s HSD

*Pattern of percentage inhibition increase is not uniform for all the microorganisms.

Structural elucidation of the Bioactive compound

IR (Infra red) analysis

The IR spectrum of 2H-Furo[2,3-H]-1-benzopyran-2-one showed absorption band in the region of 1652.9 cm^{-1} for-C-O- stretching. Further absorption bands at 1550.7cm^{-1} and 1454cm^{-1} were due to the presence of coumarin ring oxygen and furan ring oxygen respectively. The IR spectra of the bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one is as shown in Fig. 1.

^{1}H- NMR data analysis

In ^{1}H- NMR spectra, the signal due to C_3 -H and C_4-H of coumarin appeared at δ 6.41 as doublet and at δ 7.81 as singlet. The aromatic protons (C_7 + C_8) are mingled together and appeared at δ 7.7 as multiplet.

The signal due to the $C_{3'}$ and $C_{2'}$ protons appeared at 6.84 and 7.49 as doublet respectively. The NMR spectra of the bioactive compound, 2H-Furo[2,3-H]-1-benzopyran-2-one is as shown in Fig. 2.

Table 4. Treatment of different concentration of bioactive compound isolated from the seeds of *P.corylifolia* on *Aspergillus flavus* strain 6.

Microorganisms	Dry mycelial weight (gms)		Inhibition (%)
	Control	Treated	
250ppm	0.24^{b} ±0.0	0.09^{c}±1.0	55.4^{a}±1.0
500ppm	0.11^{a} ±0.1	0.04^{b} ±1.2	59.5^{b}±0.0
750ppm	0.35^{c} ±0.0	0.09^{d}±1.0	73.4^{c}±1.1
1000ppm	0.48^{d}±0.0	0.00^{a}±0.0	100.0^{d}±0.2

* Values are the mean of three replicates, ±standard error

* The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey"s HSD

* Pattern of percentage inhibition increase is not uniform for all the microorganisms.

^{13}C NMR analysis

13C- NMR data of the bioactive compound 2H-Furo [2,3-H]-1-benzopyran-2-one are recorded as solvent $CDCl_3$, δ 146.85(C_2), δ 143.8 (C_4), δ 119.77(C_3) , δ 115.5(C_7) , δ 114.76(C_8) , δ_2 106.3($C_{3'}$) and δ 99.86 ($C_{2'}$) atom (Fig. 3).

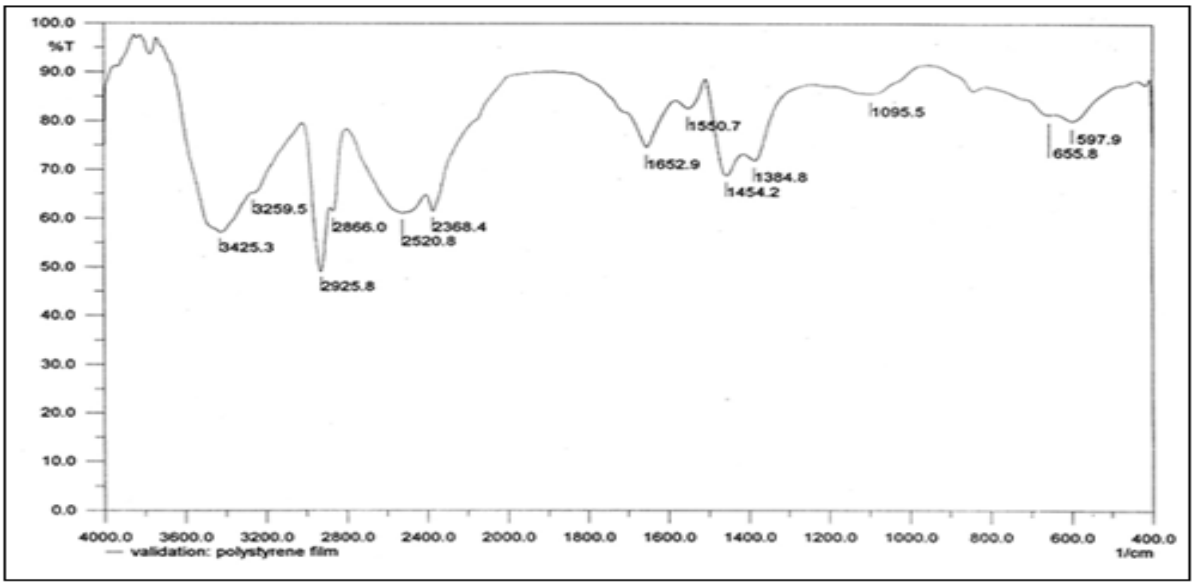

Fig. 1. IR(Infra red) spectra of the bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one, isolated form the seeds of *P.corylifolia*.

Gas Chromatography - Mass Spectroscopy (GC- MS) analysis

Showed the molecular ion peak at M/Z 186.17 consistent of molecular formula $C_{11}H_6O_3$. The peak at M/Z 158 was due to the formation of coumarin cation.

The recorded chromatogram of plot matches with chromatogram of known compound 2H-Furo[2,3-H]-1-benzopyran-2-one (Fig. 4). Fig. 5 presents the molecular structure of the bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one.

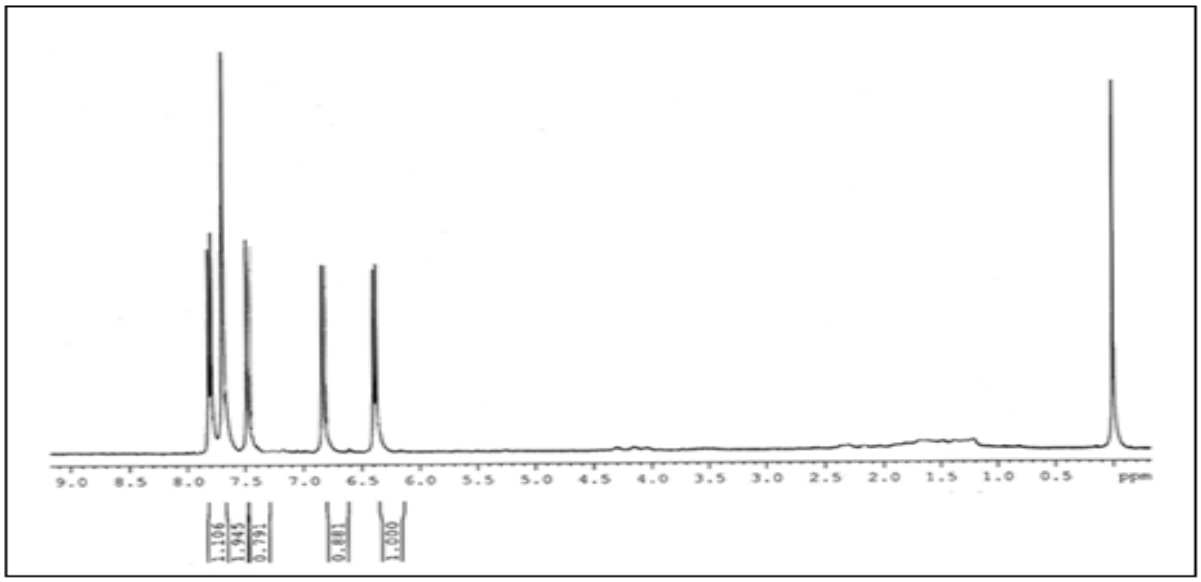

Fig. 2. ^{1}H-NMR spectra of the bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one, isolated form the seeds of *P.corylifolia*.

Treatment of different concentration of bioactive compound isolated from the seeds of P.corylifolia on Aspergillus flavus strain 6

Among the four different concentration viz., 250, 500, 750 and 1000ppm concentration of the bioactive compound tested isolated from the seeds of *P.corylifolia* on A.flavus strain 6, At 250ppm concentration, the dry mycelial weight was 0.09 grams , compared to control, it was recorded 0.24 grams .At 500ppm concentration, it was recorded

0.04 grams of dry mycelial weight and recorded 59.5% inhibition. At 750ppm concentration, the dry mycelial weight was 0.09gram and at 1000ppm concentration, the dry mycelial weight was 0.0 grams and recorded 100% inhibition. Compared to control, the dry mycelial weight was 0.48 grams (Table 4).

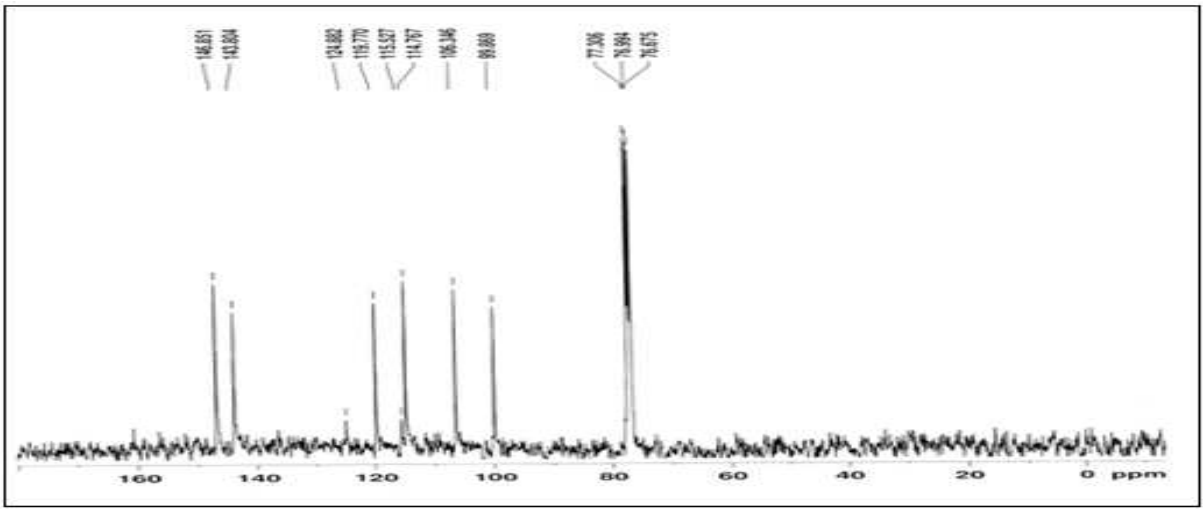

Fig. 3. ^{13}C-NMR spectra of the bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one, isolated form the seeds of *P.corylifolia*.

Extraction, Determination and Conformation of Aflatoxin B1

Extracted concentrated solution of Aflatoxin B1 from *A.flavus* strain 6 and Aflatoxin B_1 standard was subjected for Thin Layer chromatography. From the observation, it was noted that the Rf value of Standard aflatoxin was 0.56 and in extracted sample the R_f 0.56 band was completely absent. In further experiment, for the conformation of Aflatoxin B1, 2 microliter of trifluroacetic acid (TFA) was superimposed on the spot in both extracted Aflatoxin and standard aflatoxin, it was observed that, there is no movement of band in both the spots , hence confirms the presence and inhibition of Aflatoxin from the bioactive compound isolated from the seeds of *P. corylifolia*.

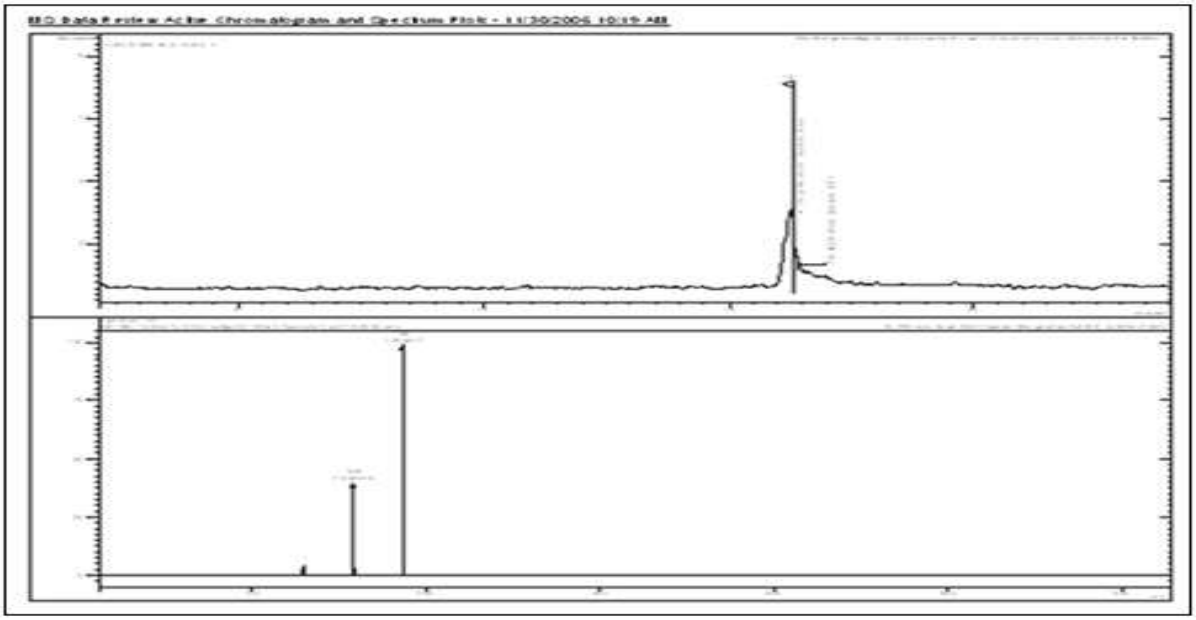

Fig. 4. Gas Chromatograph-Mass Spectrum(GC-MS) spectra of the bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one, isolated form the seeds of *P.corylifolia*.

Discussion

About 90 percent of all food crops grown on earth are propagated by seeds. Nine crops viz., wheat, rice, maize, barley, sorghum, sugar beet, bean, soybean and groundnut are of major importance and represent by far the greater part of food production in the world (Neergaard, 1997).

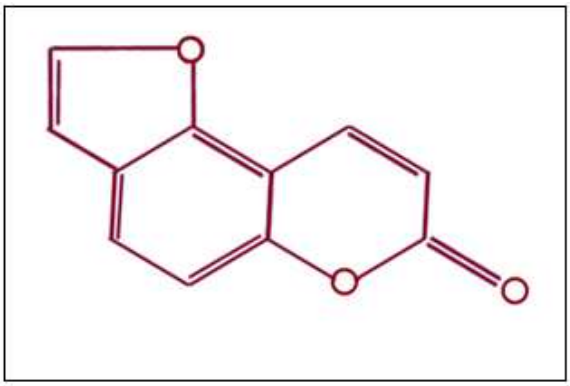

Fig. 5. Molecular Structure of the bioactive compound 2H-Furo[2,3-H]-1-benzopyran-2-one, isolated form the seeds of *P.corylifolia*.

Among the different commodities, maize ranked the top costing 66% of the total amount. USA alone detained a total of 18,000 food consignments valued $ US 1.5 billions (Bhat and Vasanthi, 1999). Plants are a repository of various biomolecules responsible for different biological activities. India is endowed with rich plant biodiversity and Karnataka is one of the hot spots of plants diversity. Many plants have been evaluated for different biological activities world over (Nair and chandra, 2006). Considering the biodiversity of higher plants, the number of plants screened for various biological activities is negligible and the number of plants screened for antifungal activities is negligible small percentage.

Further, the number of plants evaluated for antifungal activity against phytopathogenic fungi in general and biodeterioration causing fungi in particular is infitisimly small. Considering these, in the present investigation a systematic investigation was envisaged to evaluate the antifungal potency of seven plants against important biodiversity causing fungi of maize. A number of preparations made from the seeds have been tried in numerous cases of leucoderma and other skin diseases. Oral administration of the powdered seeds to the patients has generally resulted in side reactions such as nausea, vomiting, malaise, headache and sometimes purging. The seed extracts inhibit the growth of *Staphylococcus citreus*, *S. aureus* and *S. albus* including strains resistant to Penicillin. The seeds posesses anthelmintic activity against earthworms, Psoralen being the active principle (Wang *et al.*, 1999). The seeds are also used locally in the preparations of certain types of medicated oils and incense preparations.

The root is useful in the dental caries of teeth and leaves are used in diarrhoea (Katsura *et al.*, 2001). Further experiments conducted to evaluate the antifungal potency at different concentrations of aqueous extract of seeds of *P. corylifolia* revealed highly significant antifungal activity against all the test fungi in all the concentrations tested compared with control. From the literature survey, it was identified that none of researcher have reported the inhibition of aflatoxin from the biosctive compound isolated from the seeds of *P.corylifolia*. Hence in the present investigation, bioactive compound, 2H-Furo[2,3-H]-1-benzopyran-2-one isolated from seeds of *P. corylifolia* is one thing be potent plant for preventing biodeterioration of seeds by fungi.

Also the bioactive compound isolated showed a promising result in preventing and controlling the synthesis of Aflatoxin which is posing a serious health issues in humans.

Conclusion

Thus it is evident from the results of the present investigation that treatment of maize grains with 1000ppm concentration of the bioactive compound, 2H-Furo[2,3-H]-1-benzopyran-2-one isolated from seeds of *P. corylifolia* could be exploited for the prevention of moulding, mycotoxin contamination and control of biodeterioration of maize during storage. However further investigations are necessary with regard to toxicological aspects of this compound before it is finally recommended for commercial exploitation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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