J. Agr. Sci. Tech. (2014) Vol. 16: 1123-1132

Inhibitory Activity of Plant Extracts on Aflatoxin B_1 Biosynthesis by *Aspergillus flavus*

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ABSTRACT

The inhibitory activities of aqueous and solvent extracts of twelve selected medicinal plants were evaluated against biosynthesis of aflatoxin B_1 (AFB_1) by *Aspergillus flavus*. The *A. flavus* was isolated from maize, and aflatoxin B_1 biosynthesis was confirmed by comparison with standard AFB_1 using TLC method. *In vivo* antiaflatoxigenic efficacies of activity guided solvent extracts were determined in maize model system. All the extracts showed varying degree of antifungal and AFB_1 inhibitory activities, but chloroformic extract of *Albizia amara*, *Cassia spectabilis* and *Solanum indicum*, and methanolic extract of *Acacia catechu, Albizia saman* and *Anogeissus latifolia* showed the highest activity. Further investigations on identification of active principles from these plants are needed to develop plant based formulations for management of *A. flavus* growth and AFB_1 contamination in food grains.

Keywords: Antiaflatoxigenic, Maize, Plant extracts.

INTRODUCTION

Fungal deteriorations and mycotoxin contamination of various food and feedstuffs are a major problem in the tropics and subtropics, where climatic conditions and storage practices are favourable to fungal growth (Quiroga *et al.,* 2009; Shukla *et al.,* 2009; Salari *et al.*, 2012). The risk of mycotoxins, particularly aflatoxins contamination is an important food safety concern for grains and other field crops worldwide (Kumar *et al.,* 2007; Reddy *et al.,* 2009). The Food and Agriculture Organization (FAO) estimated that around 25% of the world's cereals are contaminated by mycotoxins, including aflatoxins (Dowling, 1997). Aflatoxin B_1 is one of the most common and dangerous mycotoxin produced by *A. flavus* (Manafi and Khosravinia, 2013). Aflatoxins are found in a variety of food commodities such as maize, ground nut, cotton seeds, and other cereals worldwide, and it is reported that about 4.5 billion people in developing countries are systematically exposed to uncontrolled amounts of aflatoxins (Shukla *et al.,* 2008).

The physical (aeration, cold storage, rapid drying, and radiation) and chemical (food preservatives and pesticides) treatments are commonly used to control the deterioration and aflatoxins contamination of food grains by *A. flavus* (Passone *et al.,* 2008). Most of these control strategies are costly, health hazardous, and not affordable to rural subsistence farmers (Shukla *et al.,* 2009). Further, residues of these synthetic chemicals in agricultural produce, products, and their by-products cause damage to the health of animals and humans (Deng *et al.,* 2011). Due to these, the use of natural products to control the mould and mycotoxins contamination in cereal grains, have attracted the attention of the scientists to search some newer agents from plants that inhibit aflatoxins biosynthesis. Such products of higher plants would be

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[DOR: 20.1001.1.16807073.2014.16.5.19.1]

biodegradable, renewable in nature, and safe to human health (Verma and Dubey, 1999). Different crude extracts of plant materials rich in polyphenolics and alkaloids are becoming important in food industries because of their antifungal and antiaflatoxigenic activities. Hence, such plants extracts could potentially be used to control mycotoxigenic fungi in foods and feeds, and for avoiding the use of synthetic chemicals. Considering these, we have screened 48 plants preliminarily for their inhibitory activity against *A. flavus*, among which 12 plants showed significant activity. Hence, these plants were selected for further investigations on inhibition of AFB_1 biosynthesis, and the obtained results are presented in this paper.

MATERIALS AND METHODS

Chemicals and Culture Media

The Sabouraud Dextrose Agar/Broth (SDA/SDB) and Dimethyl sulfoxide (DMSO) were purchased from Hi-Media, Mumbai (India). Mancozeb 75% WP (dithane M-45) was obtained from Indofil chemicals, Mumbai (India). All solvents, reagents and iodo-nitro-tetrazolium (INT) were procured from Sisco Research Laboratory, Mumbai (India). Microtiter plates (96 wells) and serological pipettes were purchased from Axiva, New Delhi (India). The standard aflatoxin B_1 (AFB_1) was obtained from Sigma, Germany and Silica gel 60 F_{254} coated preparative aluminium Thin Layer Chromatography (TLC) plates (20×20 cm) from Merck, Darmstadt (Germany).

Plant Materials

Fresh disease free leaves of 12 different medicinal plant species were collected from southern part of Karnataka, India. The plant samples were authenticated by Dr. Seetharam, Professor, Department of Biological Sciences, Bangalore University and the authenticated voucher specimens have been deposited at the Herbarium centre, Department of Microbiology and Biotechnology, Bangalore University, Bangalore (Table 1).

Table 1. Antifungal activity of aqueous extract of selected medicinal plants against aflatoxigenic *A. flavus* at 10% concentration.

Plants	Voucher number BUB-MB and BT-DCM-JU10-	Family	Activity (% mycelial inhibition)
Acacia catechu (L.f.) Willd.	25	Fabaceae	18.3±0.91[a]
Acacia ferruginea DC.	15	Mimosaceae	12.8±0.72
Adenanthera pavonina L.	61	Mimosaceae	12.6±0.68
Albizia amara (Roxb.) B.Boivin	23	Fabaceae	30.8±1.42
Albizia odoratissima (L.f.) Benth.	55	Fabaceae	14.5±0.85
Albizia saman (Jacq.) Merr.	33	Fabaceae	29.3±1.36
Anogeissus latifolia (Roxb. ex DC.) Wall.	24	Combretaceae	22.3±1.12
Caesalpinia coriaria (Jacq.) Willd.	44	Caesalpiniaceae	11.7±0.66
Cassia spectabilis DC.	38	Fabaceae	28.6±1.06
Dodonaea viscosa Jacq.	11	Sapindaceae	11.5±0.72
Prosopis juliflora (Sw.) DC.	12	Fabaceae	15.6±0.87
Solanum indicum L.	16	Solanaceae	42.4±1.45

[a] Data given are mean of four replicates; media impregnated with the same amount of water served as control.

[DOR: 20.1001.1.16807073.2014.16.5.19.1]

Preparation of Aqueous Extracts

The aqueous extracts of 12 plant species were prepared following the procedure of Mohana *et al.* (2007). Briefly, 50 g of thoroughly washed and blot dried plant material was macerated separately with 100 mL sterile distilled water in a warrior blender for 10 minutes. The macerate was filtered through double-layered muslin cloth, centrifuged at 4,000×*g* for 30 minutes and again filtered the supernatant through Whatman No. 1 filter paper, and sterilized at 121°C for 20 minutes. The obtained extracts were considered as 100% and 10% of each extract impregnated SDA was used for antifungal activity assay.

Preparation of Solvent Extracts

The successive solvents extracts of 12 plant species were prepared following the procedure of Thippeswamy *et al.* (2011). Briefly, 50 g powder of each shade dried plants were filled in the thimble separately and extracted successively with 200 mL of petroleum ether, toluene, chloroform, methanol and ethanol using a soxhlet extractor. The residual solvents in the extracts were removed using rotary flash evaporator. The dried plant extracts were re-suspended in DMSO and subjected to antifungal and aflatoxigenic activities at different desired concentrations.

Antifungal Activity Assay

Isolation of AFB_1 Producing *A. flavus* from Maize

A total of 45 strains of *A. flavus* were isolated from 25 maize varieties, and AFB_1 producing *A. flavus* strains were detected by methyl-*β*-cyclodextrin enriched culture media (Rahimi *et al.,* 2008). The AFB_1 content was qualitatively analysed by TLC method and quantitatively by spectrophotometric methods (Shukla *et al.*, 2008). The *A. flavus* MY5 strain was able to produce the highest concentration of AFB_1 and was selected as a test organism for determining the antifungal and antiaflatoxigenic efficacies.

Poisoned Food Technique

Aqueous and successive solvent extracts of all 12 plants were subjected to antifungal activity assay by poisoned food technique following the procedure of Mohana *et al.* (2010). Briefly, requisite concentrations of all the test samples were incorporated separately into SDA medium (10% in case of aqueous extracts and 0.031 to 4 mg mL^{-1} in case of solvent extracts), autoclaved, poured into Petri dishes (20 mL $plate^{-1}$) and allowed to cool. Five millimetre disc of 7-day-old culture of *A. flavus* was placed at the centre of the Petri dishes. The plates were incubated at 28±1°C for 7 days. The media containing DMSO served as a negative control for solvent extracts and dithane M-45 served as a positive control. Four replicates were maintained for each concentration. The fungi-toxicity of the extract in terms of percentage inhibition (I%) of mycelial growth was calculated using the following formula:

$I\% = (dc-dt)\times 100/dc$

Where, *dc*= Average diameter of mycelial growth in the control, *dt*= Average diameter of mycelial growth in the treatment.

Determination of MIC by Broth Microdilution Method

The broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) of activity guided solvent extracts following the procedure of Hajji *et al.* (2010). Briefly, 200 µL of two-fold serially diluted of each extract (0.031 to 4 mg mL^{-1}) in SDB were added separately to the 96-well microtiter plate and inoculated with 15µL of *A. flavus* spore suspension containing 10^4 spores mL^{-1} and incubated at 30°C for 72 hours. DMSO

[DOR: 20.1001.1.16807073.2014.16.5.19.1]

served as a negative control and dithane M-45 was used as a positive control. After incubation, the MIC values of the extracts were detected by the addition of 50 µL of INT (2 mg mL^{-1} in water). The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms. Where fungal growth was inhibited, the solution in the well remained clear after incubation with INT. The colour intensity was measured using microtiter plate reader (*EL$_X$800*, Bio-Tek Instruments, US). MIC was defined as the lowest concentration at which no visible fungal growth was observed.

In vitro and *In vivo* Efficacies of Activity Guided Solvent Extracts on AFB_1 Biosynthesis by *A. flavus*

In vitro Assay

The *in vitro* efficacies of activity guided solvent extracts on AFB_1 production were determined following the procedures of Shukla *et al.* (2008). Briefly, 100 µL of a spore suspension (10^4 spores mL^{-1}) of *A. flavus* was inoculated into SMKY broth containing the requisite amount of active solvent extracts (0.0312 to 2.0 mg mL^{-1}) and incubated at 28±2°C for 10 days. The flask containing medium without extract served as a negative control and dithane M-45 was used as a positive control. After incubation, the broth cultures were filtered through Whatman No. 1 filter paper and the filtrate was used for the isolation of AFB_1 by adding an equal volume of $CHCl_3$. The $CHCl_3$ layer was separated and passed through anhydrous Na_2SO_4 and allowed to evaporate in dark condition at 28±2°C. The residue was re-dissolved in 1 mL of $CHCl_3$, and 10 µL of sample was spotted on the TLC plate adjacent to AFB_1 standard. The plates were developed in $CHCl_3$-acetone (96:4) solvent system, air-dried and visualized under ultra-violet (360nm) light (UV-cabinet, Labline, India). Qualitative identification of AFB_1 content was done by visual comparison of intensity of fluorescence of the samples with AFB_1 standard spots. For quantitative estimation, the fluorescent spots were scrapped out from the plates, dissolved in 5 mL cold CH_3OH, and centrifuged at 3,000 rpm for 5 minutes. The absorbance of supernatant was measured at 360 nm using a spectrophotometer (ELICO *SL-210*, India) and AFB_1 content was calculated using the following formula:

AFB_1 content (µg L^{-1})= (DXM/EXL)×1000

Where, *D*= Absorbance; *M*= Molecular weight of AFB_1 (312); *E*= Molar extinction coefficient of AFB_1 (21,800) and, *L*= Path length (1 cm cell)

In vivo Efficacy

The *in vivo* efficacies of active solvent extracts on AFB_1 production in maize seeds were determined following the procedures of Garcia *et al.* (2012) with some modifications. Briefly, freshly harvested maize samples were collected, surface sterilized under UV, and the water activity (a_w) was adjusted to 0.95 by adding sterile distilled water. The maize samples were treated with requisite concentrations (0.0312 to 2.0 mg mL^{-1}) of activity guided solvent extracts separately and inoculated with 100 µL of a spore suspension (10^4 spores mL^{-1}) of *A. flavus*. All treatments were separately stored in plastic containers (200 g $pack^{-1}$) and incubated at 25±2°C for up to 15 days. After incubation, the milled maize seeds were subjected to AFB_1 extraction and quantification (Singh *et al.,* 1991; Shukla *et al.,* 2008).

The percent incidence of *A. flavus* in the treated and untreated samples was determined by standard blotter method (ISTA, 1996) and seedling vigour index (SVI) was analysed using the following formula (Sparg *et al.,* 2005):

SVI= (Mean of root length+Mean of shoot length)×Percentage of seed germination

[DOR: 20.1001.1.16807073.2014.16.5.19.1]

Statistical Analysis

All experiments were performed in four replicates and values were expressed as means ± standard error. Analysis of variance was conducted, and the differences between values were tested for significance by ANOVA with the SPSS 20 (IBM, USA) programme. Differences at $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The recent intensive works have revealed that the plants are important source for the development of potentially useful eco-friendly fungicides. *In vitro* evaluations are the first step towards this goal. In this study, we have screened the aqueous extracts of 12 plants *viz., Acacia catechu*, *A. ferruginea*, *Adenanthera pavonina*, *Albizia amara*, *A. odoratissima*, *A. saman*, *Anogeissus latifolia*, *Caesalpinia coriaria*, *Cassia spectabilis*, *Dodonaea viscosa*, *Prosopis juliflora* and *Solanum indicum* belonging to seven families for their antifungal efficacy in terms of percent mycelial inhibition against aflatoxigenic *A. flavus* at 10% concentration by poisoned food technique. All the plants showed varying degree of inhibitory activities with the percent mycelial inhibition ranging from 11.5 to 42.4% (Table 1). The highest percent mycelial inhibition was observed in *S. indicum*, whereas the least inhibition was observed in *D. viscosa*.

The antifungal activity of the desired different concentrations of five successive solvent extracts of each plant was determined against *A. flavus* by poison food technique for determination of percent mycelial inhibition and broth microdilution method for determination of MIC. The obtained results are presented in Table 2. The highest mycelial inhibition of *A. flavus* was observed in chloroformic extract (CE) of *A. pavonina*, *A. amara*, *C. spectabilis* and

Table 2. Inhibitory activities of activity guided solvent extracts of selected medicinal plants on AFB_1 biosynthesis and *A. flavus* growth.

Plant names	Extracts[a]	% mycelial inhibition (2 mg mL^{-1})	MIC (mg mL^{-1})	AFB_1 content[b]	
				In vitro (µg L^{-1})	*In vivo* (µg kg^{-1})
A. catechu	M	26.2±0.43[c]	1.0	300±11	850±18
A. ferruginea	M	23.8±0.16	1.0	380±12	925±22
A. pavonina	C	19.6±0.56	1.5	575±16	1325±27
A. amara	C	59.0±0.47	0.5	0	250±12
A. odoratissima	M	22.6±0.24	1.0	450±14	975±24
A. saman	M	57.8±0.72	0.5	0	250±14
A. latifolia	M	27.8±0.26	0.5	250±8	650±16
C. coriaria	M	18.6±0.52	1.0	450±12	925±23
C. spectabilis	C	42.4±0.37	0.5	100±6	425±15
D. viscosa	M	14.3±0.42	2.0	510±15	1425±28
P. juliflora	M	22.2±0.36	1.5	400±12	1010±25
S. indicum	C	63.5±0.56	0.25	0	175±12
Negative control	-	0		1500±20	2000±32
Dithane M-45	-	54.6±0.32	0.5	50±3	NC[d]

[a] P: Petroleum ether extract; C: Chloroformic extract; M: Methanolic extract, DMSO served as negative control.
[b] 2 mg mL^{-1} for *in vitro* treatment and 2 g kg^{-1} for *in vivo* treatment.
[c] Data given are mean of four replicates±standard error.
[d] NC: Not Checked.

[DOR: 20.1001.1.16807073.2014.16.5.19.1]

S. indicum, and methanolic extract (ME) of *A. catechu*, *A. saman* and *C. coriaria* with the percent mycelial inhibition ranging from 14.3 to 63.5% and MIC ranging from 0.25 to 2.0 mg mL^{-1}, depending on plant species. The *S. indicum* (CE) showed highest percent mycelial inhibition with the least MIC, whereas *D. viscosa* showed the least percent of mycelial inhibition with the highest MIC. On comparative evaluation with synthetic fungicide dithane M-45, the activity of *A. amara* (CE), *A. saman* (ME), *C. spectabilis* (CE), and *S. indicum* (CE) was comparable to the positive control dithane M-45. The present findings confirm that the chloroform and methanol are the best solvents for the isolation of bioactive compounds from the respective plants.

In vitro and *in vivo* inhibitory activities of active solvent extracts on AFB_1 biosynthesis by *A. flavus* were determined qualitatively by TLC method and quantitatively by spectrophotometric method. The results were presented in Table 2. In the negative control, AFB_1 production was 1,500 µg L^{-1} *in vitro* and 2000 µg kg^{-1} *in vivo*. The *A. amara* (CE), *A. saman* (ME) and *S. indicum* (CE) were completely inhibited the AFB_1 production *in vitro* at 2 mg mL^{-1}. Similarly, the AFB_1 biosynthesis was significantly inhibited by all of the plant species at 2 g kg^{-1} with decreased AFB_1 content ranging from 175 to 1425 µg kg^{-1}, depending on plant species. The percent incidence of *A. flavus* in maize samples of the control set was 100%, whereas, the percent incidence of *A. flavus* was greatly decreased in *S. indicum* (18.9%) followed by *A. amara* (22.5%) and *A. saman* (30.7%) treated maize (Figure 1). The present study confirms that the *A. amara* (CE), *A. pavonina* (CE), *C. spectabilis* (CE), *S. indicum* (CE), *A. catechu* (ME), *A. ferruginea* (ME), *A. odoratissima* (ME), *A. saman* (ME), *A. latifolia* (ME), and *P. juliflora* (ME) are effective extracts for inhibiting AFB_1 biosynthesis.

A survey of the literature reveals that the extracts of *A. catechu* have significant antibacterial and antifungal activities (Bhardwaj and Laura, 2009; Das *et al.,* 2011; Joshi *et al.,* 2011; Negi and Dave, 2010). Also, the antimicrobial and antioxidant activities of crude extracts of *A. amara* and *A. saman* against human and plant pathogenic bacteria and fungi have been reported (Raghavendra *et al.,* 2008; Prasad *et al.,* 2008; Azhar *et al.,* 2009;

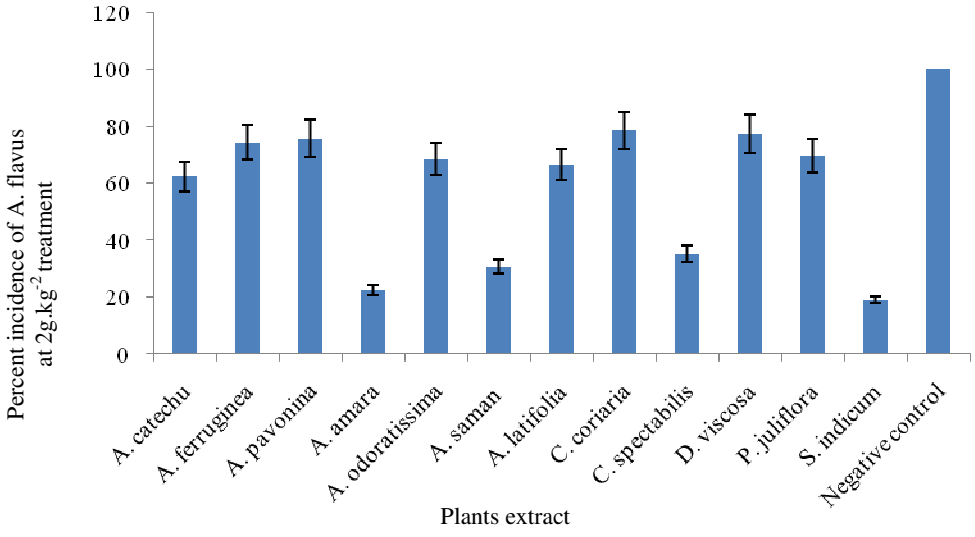

Figure 1. *In vivo* efficacy of activity guided solvent extracts of some selected plants on percent incidence of *A. flavus* in maize model system. (Data given are mean of four replicates±standard error; DMSO served as a negative control).

[DOR: 20.1001.1.16807073.2014.16.5.19.1]

Nnamdi *et al.,* 2010; Arulpriya *et al.,* 2010; Ferdous *et al.,* 2010; Praveen *et al.,* 2011; Thippeswamy *et al.,* 2011; Karmegam *et al.,* 2012; Ajam *et al.,* 2012). Other researchers have reported the anti-inflammatory, cytotoxic, and antibacterial activities of *A. pavonina* (Ahmed *et al.,* 2012, Ara *et al.,* 2010; Hussain *et al.,* 2011; Mahida *et al.,* 2007), the antimicrobial activity of the extract of *A. ferruginea*, *A. odoratissima*, *A. latifolia, C. coriaria, C. spectabilis*, *D. viscosa, S. indicum,* (Hishobkar *et al.,* 2010; Sangetha *et al.,* 2008; Ashokkumar *et al.,* 2012; Pirzada *et al.,* 2010; Siva *et al.,* 2011), and the antifungal activity of *P. juliflora* against some storage moulds (Satish *et al.,* 2007; Ikram and Dawar, 2013). To the best of our knowledge, there are no reports available on the inhibitory activity of these plants on aflatoxin B_1 biosynthesis from *A. flavus*. In the present investigation, the antiaflatoxigenic activity of these plants has been demonstrated for the first time.

The efficacy of the plant extracts over the commonly used synthetic fungicide dithane M-45 at the lowest levels of MIC with no adverse effect of treatments on seed germination with enhanced seedling growth was observed. It confirms that the collective effect of phyto-constituents of extracts may be responsible for the enhanced seedling growth. Based on the antifungal activity, the crude plant extracts could be recommended as plant-based preservatives for prevention of moulds growth and aflatoxin contamination in cereals as well as for protecting crops against fungal pathogens. This is a preliminary investigation; further studies on organoleptic parameters, and toxicological and phytochemical studies are needed before final recommendation.

CONCLUSIONS

The results of these investigations suggest that the extracts of *A. amara, A. saman, C. spectabilis* and *S. indicum* are more effective on inhibition of *A. flavus* growth and aflatoxin B_1 biosynthesis Than other plant extracts tested. Hence, these plants could be used for the development of natural fungicides for management of post harvest fungal infestation and mycotoxin contamination in food commodities after toxicological studies.

ACKNOWLEDGEMENTS

This work was financially supported by the Department of Science and Technology and the University Grant Commission, New Delhi, India.

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فعالیت بازدارندگی عصاره گیاهان روی تولید افلاتوکسین ب۱ توسط *Aspergillus flavus*

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چکیده

اثر بازدارندگی عصاره های محلول در آب یا در حلال از ۱۲ گیاه دارویی منتخب روی تولید افلاتوکسین ب۱(AFB_1) توسط *Aspergillus flavus* ارزیابی شد. *A. Flavus* از ذرت جداسازی شد و تولید AFB_1 هم با مقایسه با AFB_1 استاندارد و کار برد روش TLCبه تایید رسید. در محیط زنده، نتیجه بخش بودن اثر ضد افلاتوکسینی عصاره های محلول در حلال تحت هدایت فعالیت(activity guided) در یک سامانه مدل ذرت تعیین شد. همه عصاره ها درجات مختلفی از ضدقارچ بودن وبازدارندگی تولید افلاتوکسین ب۱ را نشان دادند ولی عصاره کلروفرمیک *Albizia amara* و *Cassia spectabilis* و *Solanum indicum*وعصار حلال در متانولیک *Acacia catechu* و *Albizia saman* و *Anogeissus latifolia* بیشترین فعالیت را نشان دادند. بررسی های بیشتر روی شناسایی ماده اصلی این گیاهان مورد نیاز است تا بتوان فرمولاسیون های گیاه-پایه برای مدیریت رشد *A. Flavus* و آلودگی های AFB_1 در بذور غلات خوراکی به دست آورد.

[DOR: 20.1001.1.16807073.2014.16.5.19.1]