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Phytochemical evaluation, acute toxicity studies and antimicrobial efficacy of seed extract of *Bixa orellana*: A plant grown in wild in Purulia district

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Abstract
Bixa orellana L. is a plant widely grown in tropics which has gained attention due to its pharmacological properties. Further it is a dye yielding plant which is obtained from the seeds which contains bixin and norbixin. Phytochemical evaluation, acute toxicity studies and antimicrobial efficacy of seed extract was conducted by deploying wide variety of protocols. Investigation revealed presence of variety of phytoconstituents such as alkaloids, flavonoids etc. Further GC MS studied showed the presence of 23 compounds some of which are phyto-estrogenic and have antioxidant properties. Antibacterial studied showed there was a clear zone of inhibition and extract was effective against both gram-positive and gram-negative bacteria when compared to broad spectrum antibiotic streptomycin. Further, the extract did not reveal any genotoxic potential at higher doses of treatment. *Bixa orellana* may be considered an important plant which has future prospects to be used against various ailments.

Keywords: Annatto, Bixa, anti-microbial, non-toxic

Introduction
Bixa orellana L. (Family: Bixaceae) is a small sized tree cultivated in the tropical regions of the world which has been widely used in food, textile and pharmaceutical industries. The seeds are also known as annato which contains a dye, a natural colorant [1-3].
The traditional healers of some ranges claim that Bixa species are more effective to treat transmittable diseases than synthetic antibiotics while the seed, root and bark were utilized as antiperiodic and antipyretic [4]. The leaves and roots are often used for treatment of epilepsy, dysentery, fever and jaundice [5].
The seeds of this plant produce one of the dyes most frequently used worldwide, not only in food products but also in the textile, paint, and cosmetic industries. Its use has been encouraged due to the prohibition on the use of synthetic dyes in food and cosmetics, where it is one of the few recognized by the World Health Organization (WHO), since, it is nontoxic.
In India, the Ayurveda practitioners use *Bixa orellana* as an astringent and mild purgative because the whole plant of it showed valuable medicinal properties and it is considered as a good remedy for treating dysentery and kidney diseases. *B. orellana* is a small tree or shrub measuring from 3 to 5 meters in height, sometimes reaching a height of 10 meters. The trunk is short, measuring 20–30 cm in diameter, with dark gray bark with lenticels in vertical rows. The leaves are alternate, 10 to 20 cm long and 5 to 10 cm wide, sharp, green on both sides, and with extended petioles while the seeds measure 0.3–0.5 cm in length and 0.2-0.3 cm in diameter, and their shape varies from pyramidal to almost conical. The number of seeds per capsule varies may contain from 30 to 60 seeds, on an average and its pericarp (layer that surrounds the seeds) contains the pigments that have wide industrial application. About 80%of this pigment is the carotenoid known as bixin, which has the dye property and can be extracted with vegetable oils or chemical bases. Depending on the cultivar and climatic conditions of the region, the bixin content can vary from 1 to 6% in the seed aril. In the food industry, it is used to colour butter, margarine, mayonnaise, sauces, mustard, sausage, soup, juice, ice cream, bakery products, macaroni, and cheese,
It is also widely used in the printing industry and dye manufacturing. Many aborigines of South America use annatto for dyeing, to colour ceramics and for other domestic use. Further, most endogenous people use this dye on their skin to beautify themselves during religious rituals and also to protect themselves from ultraviolet radiation and from other insects that infest forests [6]. Though this plant has received considerable attention with regard to its scientific basis from Latin American countries but there are few reports from Indian scientist.

This plant is found only in the dried areas of Purulia district where it is used by few tribal such as Mahalis, in painting their clothes. The seeds of the plant are often used to increase milk production and treatment of sores during summer months which was gathered from field survey of parts of Midnapore and Purulia district. This plant has received less attention in India but is grows widely in various parts of Orissa, Madhya Pradesh, parts of Bihar, Jharkhand and in Purulia district of West Bengal, hence the present investigation was carried out to find out whether aqueous seed extract of *Bixa orellana* has any toxic manifestation at higher concentration in mice model, whether it has anti-microbial properties and what phyto-constituent are present in the extract. Further, to the best of our knowledge, no investigations have been performed till date based on this plant in terms of their safety and toxic profile. Considering the medicinal significance of this plants and the need for practical data on safety, we assessed the genotoxicity and cytotoxicity studies employing *in vivo* experimental models

Materials and Methods

Preparation of the seed extract and qualitative phytochemical analysis

The seeds were collected from Purulia Ramakrishna Mission Purulia, West Bengal, India and identified. A voucher specimen has been deposited to Botany department (V-1239 MDC/2014) for record. Sundried grounded seeds obtained from pod (100 g) were extracted in various solvents such as chloroform, DMSO, ethanol and water (the ratio of plant material to solvent was 1:10 m/v). The extraction was carried out at 50 °C with constant stirring for 24 h. The extract obtained was evaporated to dryness and stored at 4 °C until required. The yield of the dried seeds was noted which was calculated by the following equation: Yield (g/100 g of dry plant material) = W1x100/W2, where W1 and W2 represented the weight of the extract after evaporation of solvent and the weight of the dried seed respectively. The presence or absence of phytochemical constituents for flavonoids, alkaloids, tannins, carbohydrates, reducing sugars, glycosides and steroids was analysed by routine procedures [7].

Ultraviolet visible absorption analysis (UV)

One g of BO seed powder was kept overnight with 25 ml of various solvents (water, ethanol, DMSO, Chloroform) with constant stirring and then filtered. An aliquot of the filtered sample was scanned using UV-visible Spectrophotometer (Shimadzu, UV-1800), at a range of 190-450 nm (scanning speed-medium, and slit width 1), to detect the characteristic wavelength of the extracts.

GC/MS analysis of *Bixa orellana* seeds

GC/MS analysis of only aqueous BO seeds was conducted by Gas chromatograph coupled to a mass spectrophotometer equipped with a fused capillary column, Model No: Agilent 190915-433 (HP-5MS, 0.25 mm × 30 m × 0.25 µm). For GC/MS detection on electron ionization system with ionization energy of 69.9 eV was utilized. The carrier gas was Helium (99%) with a constant flow rate of 1 mL/min. The volume of the sample injected was 5 µL in GC grade ethanol with an average velocity of 37 cm/sec. The initial temperature of column was 50 °C for 5 min and then, it was programmed to 280 °C. The total GC running time was 28 min.

Acute toxicity studies

For acute toxicity studies we took mice (*Mus musculus*) 22-25 g of either sex and treated with 500mg/kg body weight of aqueous extract determined the chromosomal aberrations and mitotic indices and compared it with normal control at 48 and 72hour fixation interval. For this, mice were intra-peritoneally injected with 0.025% colchicine at 1 ml/100 g bw 1.5 h before sacrifice and slides were prepared by the conventional flame-drying technique followed by Giemsa staining for scoring chromosome aberrations in the bone marrow [8-9]. A total of 100 bone-marrow cells were observed, from each of the four mice in a treatment group. For the mitotic indices study part of the suspension of bone-marrow cells was smeared on clean grease-free slides, briefly fixed in methanol and subsequently stained with May-Grunwald followed by Giemsa. The mitotic index was assessed by calculating the ratio of non-dividing and dividing cells. The animal experimentation was performed under the supervision of IAEC, Sidho-Kanho-Birsha University (1973/GO/Re/S/17/CPCSEA).

Study of antibacterial activity

The antibacterial activity of the water extract of *B. orellana* was investigated by standard agar-well diffusion method into three Gram positive (*Bacillus subtilis, Bacillus cereus, P. mirabilis*) and one Gram negative (*Proteus mirabilis*) bacteria [10]. Briefly, nutrient agar solidified onto petri plates, and the plates containing nutrient medium were evenly inoculated with 100 µg (108 CFU/ml) separately. The wells are prepared on the agar plate with the help of cork borer (0.6 cm diameter). Streptomycin, 5 µg /disc, was used as standard, was placed in the well of each plate. The water extract containing (10 µg /ml) was loaded onto the wells of each plate. The plates were then incubated for 24 hour at 37 °C, and the antibacterial activity was determined by measuring the diameter of the inhibition zone and expressed in millimetre.

Statistical Analysis

All the qualitative test/analysis was performed in triplicate. The statistical significance of difference between data of the different groups was calculated by Students't-test. ANOVA (SPSS 10.0 Software) was used to compare multiple groups and differences within the groups.

Results

The pod and seeds of *B. orellana* is represented in figure 1 and the percentage yield of the extract was 17%.

Fig 1: Pods and seeds of B. Orellana which were collected in wild

The qualitative test for different phytochemicals in aqueous seed extract are represented in table 1 which showed presence of flavonoids, alkaloids followed by steroids, tannin,

glycosides and then by carbohydrates, reducing sugars (+ sign indicates the intensity of their presence).

Table 1: Phytoconstituents present in the seed extract if *Bixa orellana*

Chemical compounds	Seed extracts of BO
Flavonoids	++++
Tannin	+++
Carbohydrate	++
Reducing sugars	++
Glycosides	+++
Steroids	+++
Alkaloids	++++

UV Vis analysis of aqueous extract of the BO seeds revealed that there is a single major peak at 400 to 500nm when scanned from 200 to 800nm with a large range of peak area (Figure 2). When DMSO extracted seeds were subjected to 200 to 800 nm scanning with uv-vis it revealed 3 major peaks at 250nm and at 472 and 502 nm. These two peaks were mainly of presence of the colouring materials present in the seed extract norbixin and bixin (Figure 3). When chloroform extract was subjected to Spectrophotometric analysis it revealed 3 major peaks at 248nm, 472nm and 500nm, these two peaks is due to bixin and norbixin which was similar to DMSO extract (Figure 4, 5).

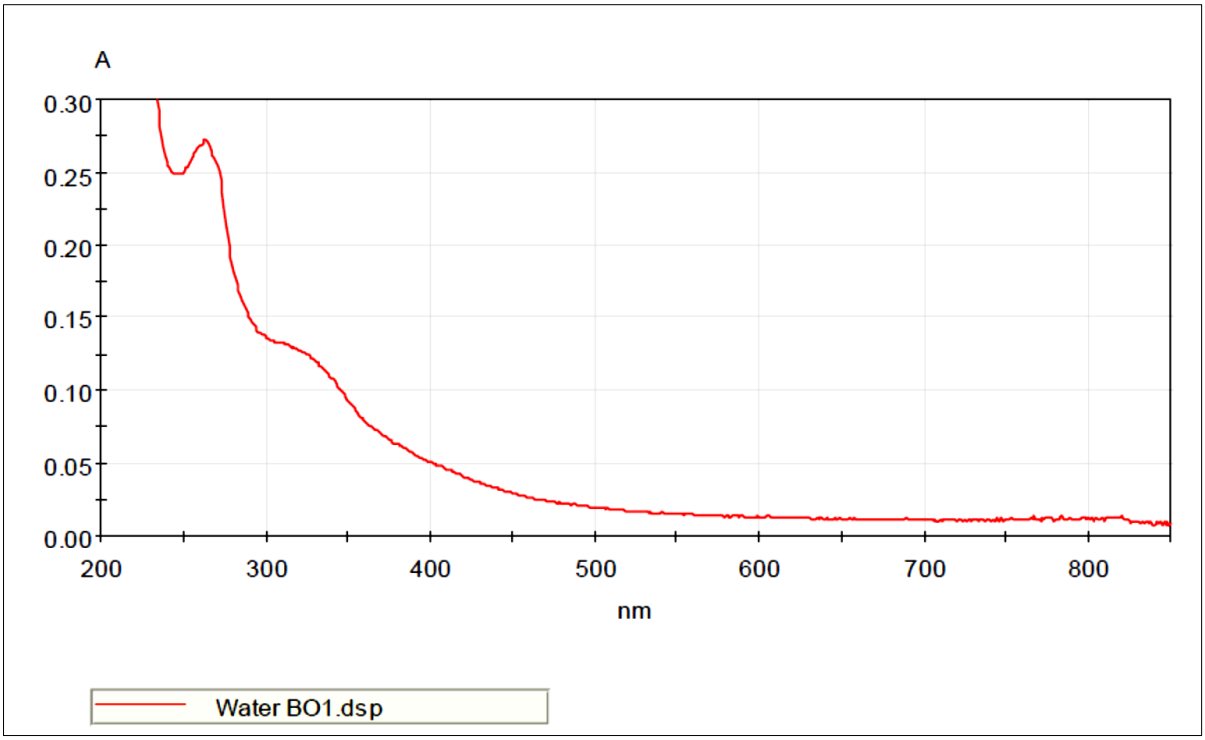

Fig 2: UV-VIS analysis of aqueous seed extract

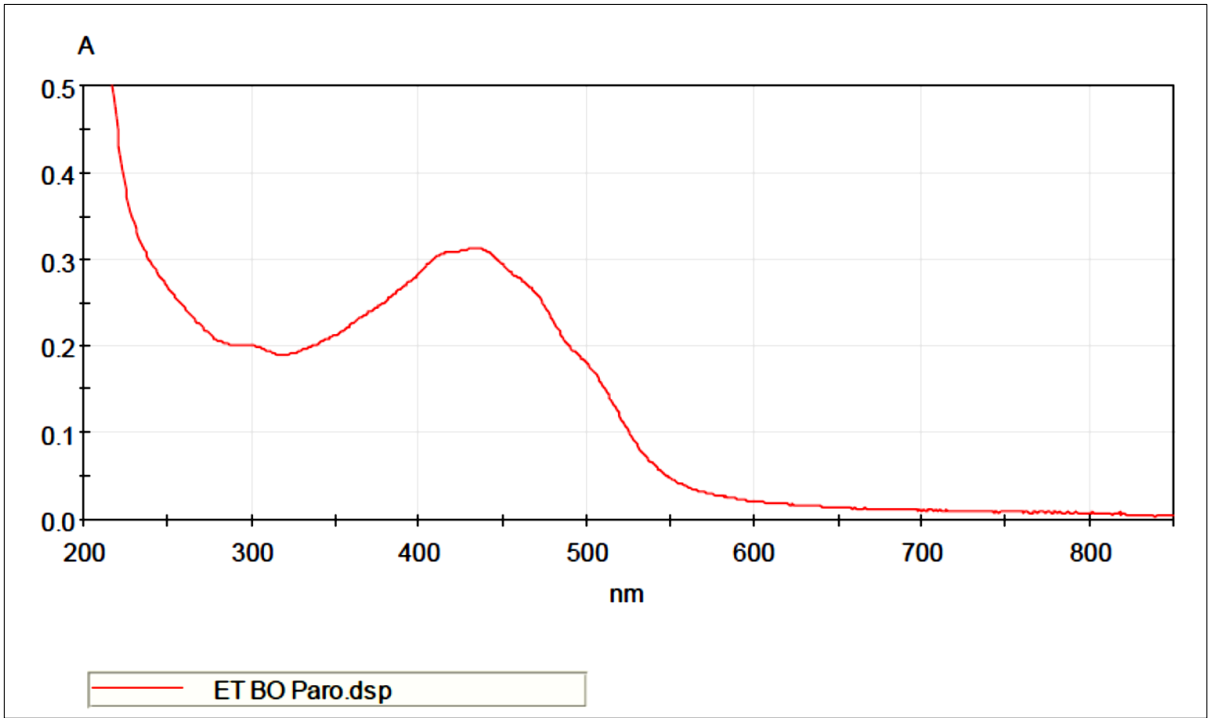

Fig 3: UV-VIS analysis of aqueous seed extract

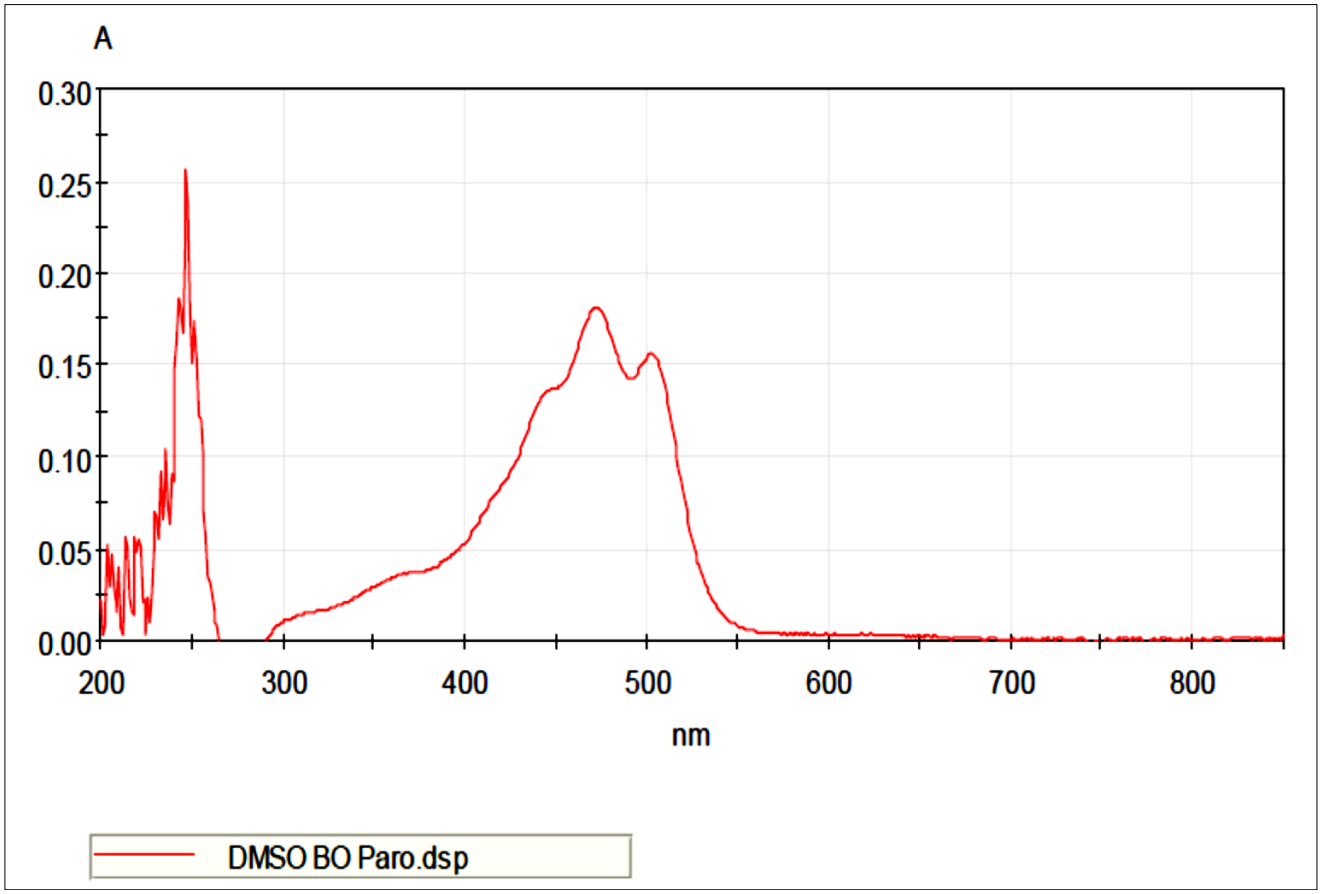

Fig 4: UV-VIS analysis of seed extract in DMSO

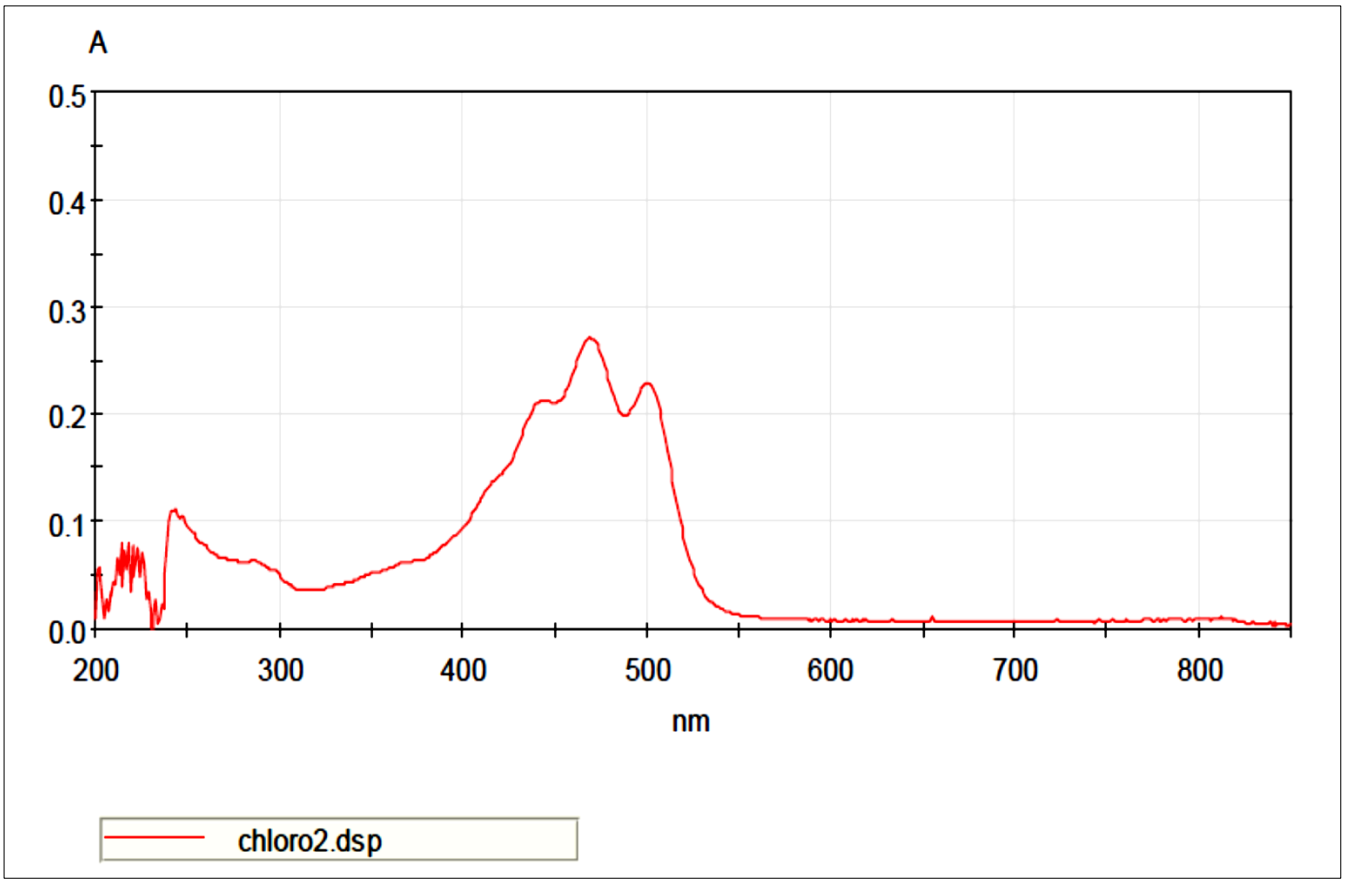

Fig 5: UV-VIS analysis of seed extract in Chloroform

GC-MS analysis of the aqueous seed extract reveals presence of 23 compounds see Table 2. Some of the compounds have huge peak area such as α-Guaiene of 25.29, α-Farnesene of 17.96. There are few compounds present which have antioxidant properties such as β-Sitosterol, caryophyllene, ambrein, some of the compounds are phyto-estrogenic.

Table 2: Showing GC-MS analysis of the aqueous seed extract of BO, the compounds present with their retention time and peak area

No	RT	Name of the Compound	Mol. Formula	M. Wt	Peak area
1	5.47	Benzoic acid	$C_{19}H_{10}O_2$	150	0.26
2	8.11	Alfa Copaene	$C_{15}H_{24}$	204	0.39
3	8.74	Aristolene	$C_{15}H_{24}$	204	0.6
4	9.13	Azulene	$C_{15}H_{24}$	204	0.98
5	9.31	Megastigma-&E, 9,13-triene	$C_{13}H_{20}$	176	0.87
6	9.5	α-Guaiene	$C_{15}H_{24}$	204	25.29
7	9.66	Isoledene	$C_{15}H_{24}$	204	1.99
8	9.76	Guaia-1(10), 11-diene	$C_{15}H_{24}$	204	2.05
9	10.06	δ-Cadinene, (+)-	$C_{15}H_{24}$	204	2.87
10	10.85	α-Gurjunene	$C_{15}H_{24}$	204	1.22
11	11.51	β-Vatirenene	$C_{15}H_{22}$	202	3.25
12	12.85	Ledene-oxide-(II)	$C_{15}H_{24}O$	220	0.13
13	12.42	4,5-di-epi-aristolochene	$C_{15}H_{24}$	204	1.01
14	14.78	α-Springene	$C_{20}H_{32}$	272	1.20
15	15.5	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	2.89
16	17.97	9,12-Octadecanadienoic acid methyl ester	$C_{19}H_{34}O_2$	294	2.88
17	18.04	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	$C_{18}H_{30}O_2$	278	2.94
18	18.57	α-Farnesene	$C_{15}H_{24}$	204	17.96
19	20.06	Caryophyllene	$C_{15}H_{24}$	204	2.49
20	31.28	Ambrein	$C_{30}H_{52}O$	428	22.81
21	33.39	Lupan-3-ol, acetate	$C_{32}H_{54}O_2$	470	2.59
22	35.28	Stigmasta-5,22-dien-3-ol, acetate, (3β)-	$C_{31}H_{50}O_2$	454	1.22
23	36.91	β-Sitostserol	$C_{29}H_{50}O$	414	2.13

On analysis of the extract of BO seeds in *in vivo* conditions after 48 and 72 hours treatment of mice did not reveal much change with that of the controls. Though we encountered few aberrations when compared to normal control even at a dose of 500mg/kg weight of the animal but it was not statistically significant. Also, there was not much variation in the mitotic indices in the treatment group when compared to normal control. (Figure 6, 7 and Table 2).

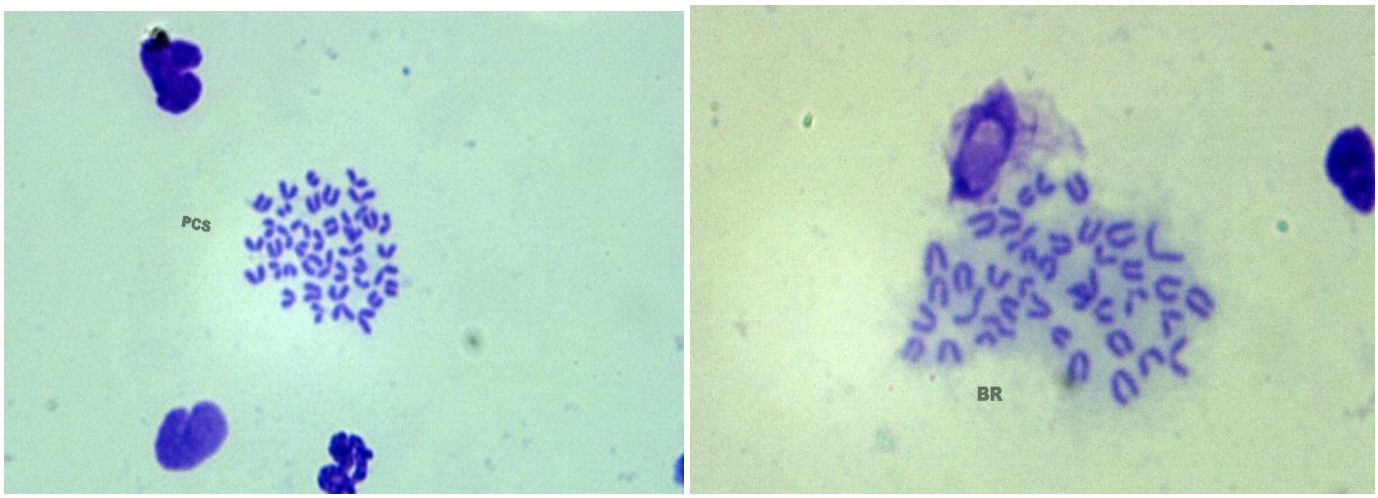

Fig 6-7: Representative bone marrow chromosome aberrations of mice treated with 500mg/kg body weight of the extract for 48 and 72 hours

Table 3: Showing Percentage aberration and mitotic indices at 48hour fixation intervals

Interval	Series	% Chromosomal aberration	%Mitotic indices
48 hr	Normal Control	0.46±0.74	1.21±0.47
	Only DW treated	0.50±0.22	0.92±0.07
	BO treated 500mg/kg b. wt	0.52 ± 0.08^{n}	0.95 ± 0.41^{n}
72 hr	Normal Control	0.55±0.04	1.01±0.22
	Only DW treated	0.59±0.11	0.92±0.07
	BO treated 500mg/kg b. wt	0.61 ± 0.07^{n}	1.18 ± 0.11^{n}

Antibacterial activity of seed extract of one Gram negative bacteria *Proteus mirabilis* and three Gram positive bacteria *Bacillus subtilis, S. aureus, B. cereus* is shown in Figure 8-11. In most of the cases there was a clear zone of inhibition. The extract was effective against both gram-positive and gram-negative bacteria when compared to broad spectrum antibiotic streptomycin. Figures also showed that in case of *B. cereus* the zone of inhibition was less when compared to other gram positive bacteria *B. subtilis S. aureus* and the activity index % was also low.

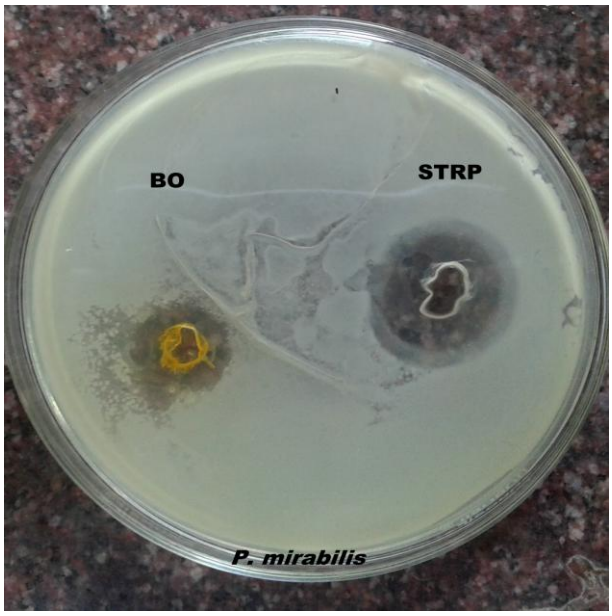

Fig 8

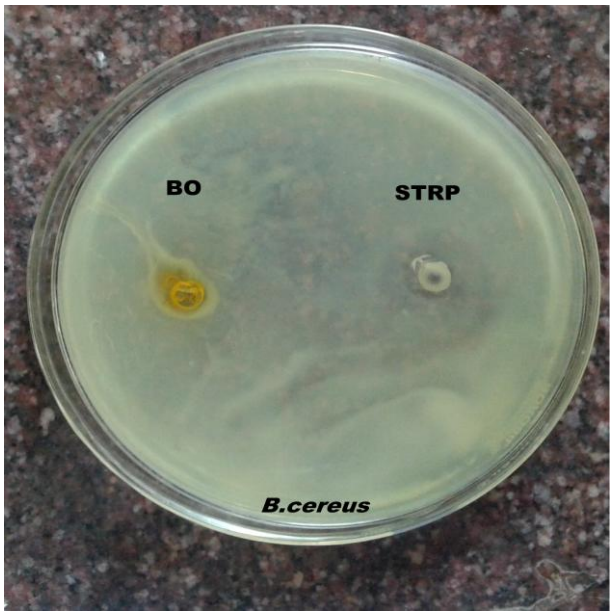

Fig 10

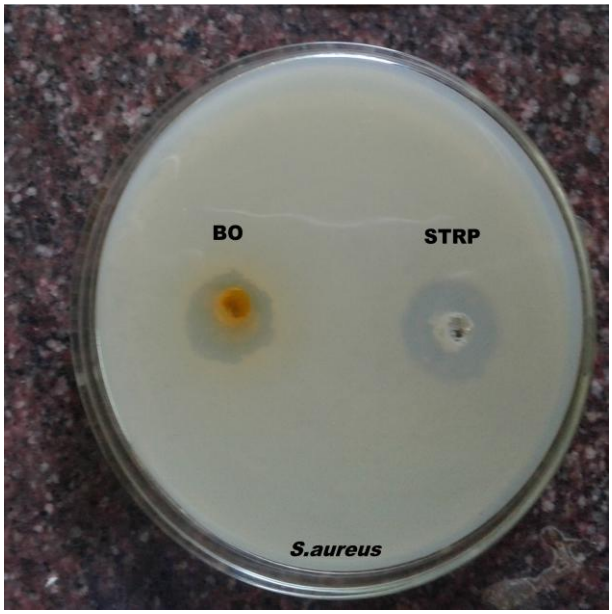

Fig 9

Fig 11

Table 3: Table showing percentage activity index of aqueous seed extract of BO against 3 gram (+) and one gram (-) bacteria

S. No.	Microorganism	Inhibition zone (mm)	Activity index %	Standard antibiotics (mm)
1.	*Bacillus subtilis (+)*	17.21±1.11	18.89	21.22±0.24
2	*Bacillus cereus(+)*	14.86±1.21	7.81	16.12±0.18
3	*S. aureus(+)*	17.24±0.22	14.9	20.26±0.18
4	*P. mirabilis(-)*	15.15±0.25	32.57	22.47±0.47

Discussion

The annato seeds traditionally are often used in the form of decoctions, juices for the treatment of common infections, to cure skin diseases, wounds and burns [11]. The leaves are also used as febrifuge in parts of South East Asian countries. It has been reported by others that decoction of leaves is used for treatment of dysentery and often prescribed as a purgative. Further, the oil obtained from the seeds is used to cure leprosy and jaundice. In Ayurvedic form of medicines it is used as an astringent and considered as a decent remedy for treating dysentery and kidney diseases. Wong *et al*. 2018 reported that tocotrienols from BO has potential to manage osteoporosis due to its ability to prevent hormonal changes and thereby preventing bone loss [12]. Further, it has been reported by others that topical application of bixin protects against photodamage by activating a transcription factor NRF2 [13]. Xu *et al*. 2017 reported that bixin present in BO seeds might be helpful in supressing fibrosis, inflammation and oxidative stress which in turn reduces high fat diet induced cardiac injury [14]. It has been reported that annatto is not carcinogenic neither maternally toxic nor embryotoxic which is in line with the present investigation conducted by us [15-16]. Some investigators showed no lethal effects within 24h after the administration of annatto extract, even at the highest dose tested (4,000 mg/kg) for mice [17]. In the present investigation we found that the antibacterial activity of BO extract also flavonoids and alkaloids were more therefore we can conclude that these flavonoids have the capability to complex

with proteins and with bacterial cell walls and lipophilic flavonoids may also disrupt bacterial membranes [18]. Even at higher doses it did not reveal any chromosomal abnormality when compared to normal and positive control therefore it would be subtle to say that even at higher doses it is safe and does not produce cytotoxicity.

The experiment concluded that the extracts contained numerous active phytoconstituents which might be responsible for their biological activity. Such studies can generate interest in others and can lead to discovery of new drugs for the betterment of human population.

Acknowledgements

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