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STUDY ON CLOSTRIDIUM PERFRINGENS TYPE A INFECTION IN BROILERS OF WEST BENGAL, INDIA

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ABSTRACT

This study was conducted to report the incidence of Clostridium perfringens Type A in the broiler chicken, poultry feed and farm environment and also to find out the most effective antibiotic on the isolates. A total of 900 samples comprising of 400 samples from dead broiler birds, 150 samples from compound poultry feed, 150 samples of poultry feed components of animal source origin and 200 samples of farm environment (litter, wall swab and water) were collected from different poultry farms of North 24 Parganas, Kolkata, Howrah and Nadia districts of West Bengal (India) and were processed for the isolation of the organism and finally, the isolates were tested for antibiotic sensitivity. The result showed that the incidence rate was higher in compounded poultry feed (59.33%) followed by poultry feed components of animal origin (54%), farm environment (53%) and dead broiler birds (50.25%) indicating the chance of establishing infection among broiler birds clinically or sub-clinically. The antibiotic sensitivity test revealed that Penicillin-G is the most effective drug against the isolates of C. perfringens Type A, whereas gentamicin, streptomycin, kanamycin and tetracycline were found ineffective suggesting the use of antibiotics after proper investigation of drug resistance.

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KEY WORDS

Antibiogram, Broiler chicken, Clostridium perfringens Type A, Incidence

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[I] INTRODUCTION

Clostridium perfringens Type A is a bacterial pathogen causing necrotic enteritis in broiler birds and is responsible for causing both visible and invisible economic losses through mortality, morbidity, weight loss, low feed conversion ratio and poor performance. *C. perfringens* Type A is also associated with other poultry diseases including avian malignant gas edema [1], gizzard erosion [2] and gangrenous dermatitis [3]. A high number of *C. perfringens* Type A in the intestinal tract and associated necrotic lesion have been detected world wide in poultry flocks that suffer from necrotic enteritis [4, 5]. To combat the disease as well as to minimize the losses due to this bacterial infection in poultry, proper investigation, and identification of the organism and antibiogram studies are very essential. Keeping in view these fact, the present work was planned to predict the prevalence of *C. perfringens* Type A in poultry feed and farm environment and also to find out the most suitable antibiotics to be effective on the isolates.

[II] MATERIALS AND METHODS

A total number of 900 samples amongst which 400 samples from dead poultry birds of 2-6 weeks age having the history of enteritis, diarrhea and low feed conversion ratio and frequent mortality; 150 samples from compound poultry feed i.e. broiler starter, finisher, developer pellet feed and poultry feed containing vegetable protein; 150 samples of poultry feed components of animal source origin like fish meal, meat cum bone meal, meat meal and blood meal and 200 samples of litter, wall swab and water were collected from different poultry farms of North 24 Parganas, Kolkata, Howrah and Nadia districts of West Bengal (India). These samples were processed for the isolation of *C. perfringens* Type A as per the method described by Cruickshank et al. [6] and Balows et al. [7]. Cooked meat medium (RC medium) and tryptose sulfite cycloserine (TSC) agar, Perfringens agar were used for isolation and identification on the basis of cultural, morphological and biochemical characteristics. Perfringens agar was used for the susceptibility test with 24 h incubation period [8]. Overall, randomly 100 isolates of *C. perfringens* Type A were tested for antibiotic sensitivity against antibiotics viz., gentamycin, kanamycin, neomycin, streptomycin, tetracycline, amikacin, cotrimoxazole, cloxacillin, amoxicillin, ampicillin, lincomycin, chloramphenicol, metronidazole, erythromycin and penicillin-G using the disc diffusion method [8].

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[III] RESULTS

Overall, 477 (53%) isolates of Clostridium perfringens Type A were recovered from the total 900 samples of which 201 (50.25%) from dead broiler birds, 89 (59.33%) from compounded poultry feed, 81 (54%) from poultry feed components of animal origin and 106 (53%) from farm environment. The percentage of incidence / prevalence was higher in compounded poultry feed followed by poultry feed components of animal origin, farm environment and dead broiler birds **[Table -1]**. Out of 400 samples (intestine and liver) of dead broiler birds, 201 (50.25%) isolates of *C. perfringens* Type A were recovered. In compounded poultry feed, out of 50 samples of broiler starter, 50 samples of broiler finisher, 25 samples of developer pellet and 25 samples of poultry feed containing vegetable protein yielded 36 (72%), 30 (60%), 13 (52%) and 10 (40%) isolates of *C. perfringens* Type A respectively. Among the farm environmental samples in the form of litter (100), wall swab (40) and water (60), isolates of *C. perfringens* Type A was found in 65 (65%), 21(52.5%) and 20(33.33%) samples respectively.

The results of antimicrobial sensitivity test revealed that 78% isolates were sensitive to penicillin-G followed by chloramphenicol (76%), lincomycin (73%), ampicillin (67%), metronidazole (60%), amoxycillin (57%), cloxacillin (5%), erythromycin (50%), cotrimoxazole (40%), neomycin (20%) and amikacin (8%). some isolates were intermediately sensitive to erythromycin (15%), penicillin-G (12%), neomycin (10%), chloramphenical (9%), lincomycin (7%), kanamycin (5%) and amikacin (2%). All the isolates (100%) were found resistant to gentamicin, streptomycin and tetracycline.

Table-1: Incidence of *Clostridium perfringens* Type A in various samples

Source	Sample	Number of samples collected	No. of samples positive for *C. perfringens* Type A	Sample wise of incidence percentage (%)	Total number of samples positive for *C. perfringens* Type A from each source	Percentage of incidence (%) from each source
Dead poultry birds	Intestine, liver	400	201	50.25	201	50.25
Compounded feed	Broiler starter	50	36	72	89	59.33
	Broiler Finisher	50	30	60		
	Developer pellet	25	13	52		
	Poultry feed containing vegetable protein	25	10	40		
Poultry feed from animal origin	Fish meal	50	27	54	81	54
	Meat cum bone meal	50	26	52		
	Meat meal	35	20	57		
	Blood meal	15	8	53.33		
Farm environment	Litter	100	65	65	106	53
	Wall swab	40	21	52.5		
	Water	60	20	33.33		
TOTAL		**900**	**477**	**53**	**477**	**53**

[IV] DISCUSSION

The finding in the present study fully corroborates with the findings of Hussein and Mustafa [9]. The incidence of *C. perfringens* Type A in the environmental samples in the present study was close to the observation of Carven [10].The results depicted in the present study were similar to the findings of Kohler [11] who also isolated 72 isolates of Clostridium perfringens from 102 compounded feed samples. In poultry feed components of animal origin, 50 samples of fish meal, 50 samples of meat cum bone meal, 35 samples of meat meal and 15 samples of blood meal yielded 27 (54%), 26 (52%), 20 (57%) and 8 (53.33%) isolates of *C. perfringens* Type A respectively. Similar findings were also reported by Komnenov et al. [12] and Pupavac and Lalic [13].

The present findings supported the observation of Hussein and Mustafa [9] and Ibrahim et al. [14]. Higher sensitivity to penicillin-G and complete resistance to Streptomycin and Tetracycline was also recorded earlier [15]. However, there are reports of sensitivity of *C. perfringens* type-A to tetracycline [16].

100% resistance of the isolates to the tetracycline observed in the present study may be attributed to difference in isolates of *C. perfringens* Type A as determinant of resistance is frequently

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transferable through plasmid salt transfer and plasmid mobilization [17]. Das et al. [18] studied the drug resistance pattern of *C. perfringens* type-A isolated from dead broiler and layer birds in Assam and West Bengal and recorded 100% efficacy of benzyl penicillin, chloramphenicol, erythromycin, lincomycin and metronidazole against the isolates. This variation of the sensitivity of the antimicrobial agents may be due to different antigenic constituents of *C. perfringens* Type A. Again, such differences in sensitivity may be attributed to the frequent use of antimicrobials for treatment and / or as a growth promoter in the feed.

[VI] CONCLUSION

Clostridium perfringens Type A was found to be wide spread in the poultry farm environment including poultry feed and among broiler birds infected clinically or sub-clinically in West Bengal, India. It is also evident that Penicillin-G can be the drug of choice in *C. perfringens* Type A infection in broiler birds whereas gentamicin, streptomycin, kanamycin and tetracycline were found ineffective. So, indiscriminate and injudicious use of antibiotics/antimicrobial agents should be restricted and they must be used after a proper investigation of drug resistance.

C. perfringens is frequently found in the intestinal tract of healthy poultry. Isolation of *C. perfringens* may not show active infection sometimes Nectotic enteritis B like toxin (NetB) is critical virulence factor in the pathogenesis of necrotic enteritis in broilers. Virulence factors in the isolates have not been investigated in the present study.

CONFLICT OF INTERESTS

The authors declare no competing interest in relation to work.

FINANCIAL DISCLOSURE

Nil

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BIO-AUTOGRAPHY: AN EFFICIENT METHOD TO CHECK THE IN VITRO ANTIMICROBIAL ACTIVITY OF *AEGLE MARMELOS* AGAINST ENTERIC PATHOGENS

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ABSTRACT

Aegle marmelos Correa (Rutaceae) is an indigenous plant of India. It is used in traditional medicine for a variety of purposes, in treating gastrointestinal disorders, especially diarrhea caused by enteric organisms. The great future potential of this plant has created a need for an antimicrobial assay, which can effectively confirm its antimicrobial activity. The objective of this study was to identify and confirm the compounds in the methanolic extract of fruit pulp of Aegle marmelos having antimicrobial effect against multi drug resistant clinical pathogens isolated from stool samples, by the bio-autography technique. The results obtained indicated that the compound with Rf value 0.71 and 0.75 respectively showed antibacterial activity in the methanolic extract of fruit pulp of Aegle marmelos against Salmonella typhi B330. Further, on purification and crystallization, the results could be validated. These two compounds, to the best of our knowledge, have shown antimicrobial activity for the first time by this method. Hence the method has potential in determining the efficacy of medicinal plants against other clinical pathogens as well.

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KEY WORDS

Traditional; diarrhea; enteric; assay; multidrug resistant

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[I] INTRODUCTION

In Asia, Africa and Latin America, gastrointestinal disorders are the leading cause of childhood mortality, resulting in 4,600,000 to 6,000,000 deaths each year. At the same time, diarrhoea is a common adverse effect of antibiotic treatment [1], which results from growth disruption of normal micro flora of the gut. The World Health Organization [2] attributes 3.5 millions deaths a year to diarrhea, with 80% of these deaths occurring in children under the age of five years and most often occurring in children between six months and three years of age. In Indian children, diarrhoea is the most common ailment (73%), and *Aegle marmelos* has been often used to cure diarrhoea and dysentery [3].

L. Correa (Rutaceae] known as "Bael" is an important indigenous fruit of India [4], and has great mythological and religious significance. It is called "shivadume", the tree of Lord Shiva. It is a large deciduous tropical tree, found all over India in Sub-Himalayan forests, Bengal, Central and South India and also in Burma. Since ancient times, its leaves and fruits are offered to Lord Shiva and Parvathi [5]. Ayurvedic physicians in India use almost all of its parts in many indigenous plant preparations. Extracts of *Aegle marmelos* are associated with various medicinal properties [6]. The unripe fruit is used in diarroheal and intestinal conditions and contains marmelosin (furocoumarin) as a chief constituent. It also contains carbohydrate, pectin, volatile oils and tannins and possesses astringent, digestive and stomachic actions and is useful in chronic diarroheas [7].

Due to its high pectin content, it is useful in diarrhea and dysentery and its volatile oils and tannins possess astringent properties [8-9]. In some studies, the unripe fruit showed activity against some intestinal parasites [10-11]. The plant has also shown to have antibacterial and antifungal activity [12-13]. The aqueous extract of its seed is hypoglycemic [14]. The ripe fruit has been shown to be effective against Ranikhet Disease virus, human Coxsackie virus B1-B6 [15] and in chronic dysentery and gastrointestinal diseases [16-18]. Its biological actions included antimicrobial, anthelminthic and antifilarial activities [19]. Aqueous extract of *Aegle marmelos* enhanced the susceptibility of beta-lactam resistant *Shigella flexneri* and *Shigella dysenteriae* towards beta-lactam antibiotics by altering porin channels [20]. As per Charaka (1500 BC], no tree has been in fact, longer or better known or appreciated by people of India than the Bael [21].

Aegle marmelos has also been widely investigated for its phytochemical constituents. The root, stem and leaves have been shown to contain tannins, alkaloids, sterols, coumarin, phenyl ethyl cinnamides and aromatic components [22, 23].

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Aegelin, marmelosine, marmelin, o-methyl hayordinol, alloimperatorin methyl ester,o-isopentanyl hayordinol and linoleic acid have been identified as its secondary metabolites [24-26] . The dry pulp of the fruit contains chiefly mucilage pectin like substance. Umbelliferone, psoralen and eugenol have been isolated from the dried fruit pulp and quantified by HPTLC [27]. Antimicrobial activity has also been shown in the seed oil against bacteria and fungi [28]. Analgesic, hypoglycemic, antioxidant and hepatoprotective effect of fruits and leaves has also been reported [29-31]. We report here, the results obtained using bioautography to detect antimicrobial compounds in a fruit pulp of *Aegle marmelos*, a plant used widely in traditional medicine for the treatment of diverse infectious diseases.

[II] METHODS

2.1. Plant material

Aegle marmelos fruit was collected in and around Chandigarh. The identity was established with the help of Department of Botany, Panjab University, Chandigarh.

2.2. Preparation of plant extracts

Two types of extracts [methanolic and aqueous] were used. Extracts were prepared by stirring the plant material overnight at room temperature with a seven-fold amount of water/methanol. The suspension was cold centrifuged at 3000 rpm for 15-20 minutes and the supernatant collected. The suspension was filtered through a Whatman filter paper No. 1, concentrated to half of its original volume at room temperature, filter sterilized with 0.45 µm Millipore filters and stored in screw capped vials at –20^{0}C [32].

2.3. Microorganisms used

Clinical isolates of *Salmonella typhi* from patients with enteric diseases were procured from Govt. Medical College and Hospital, Chandigarh. The antibiotic resistance pattern was studied by the disc diffusion method using standard Octodiscs [Hi media, Pvt. Bombay, India]. The zone of inhibition was measured after incubation at 37^{0}C for 24 hrs. In addition, a standard strain of *Salmonella typhi* [MTCC-531] was procured from the Institute of Microbial Technology, Chandigarh.

2.4. Extraction and fractionation of components from the fruit pulp of A.marmelos

The fresh mashed fruit pulp [500gm] of *Aegle marmelos* was macerated two times with methanol [[1.5 L×24h, 1.5 L×24h]] at room temperature. Each time, the extract was filtered and solvent removed under vacuum in rotatory evaporator below 50^{0}C to obtain a syrupy residue. The residue was combined to get 10.2gm of methanolic extract **[Figure– 1]**.

2.5. Fractionation using column chromatography

The residue [10.2gm] was subjected to chromatography over silica gel [150 gm, 150- 200 mesh] packed in petroleum ether [60-80^{0}C]. The column was eluted using acetone and petroleum ether [60-80^{0}C] with increasing portions of acetone [petroleum ether, PE+10% acetone, PE+30% acetone, and PE+50% acetone, acetone]. Finally the column was eluted with acetone. A total of 10 fractions, of 250ml each were collected and pooled according to their similarity to analytical TLC plates and dried. The different fractions thus obtained were dehydrated and concentrated in a rotary evaporator at 40°C. The active fraction was selected for isolation and purification of the antimicrobial compound.

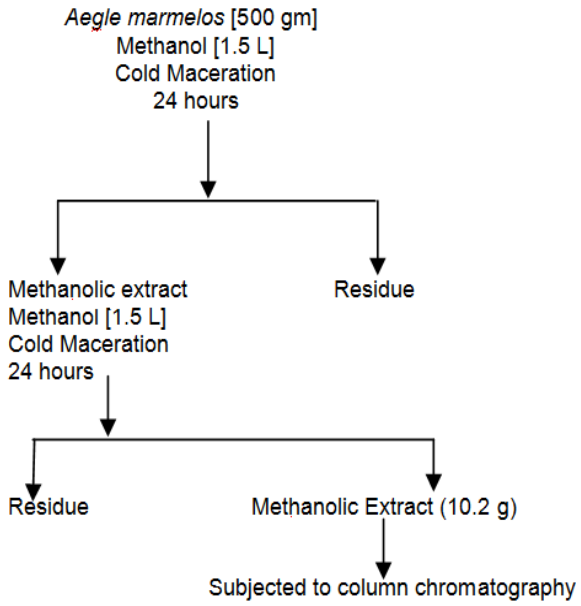

Fig: 1. Extraction and fractionation of *A. marmelos* fruit pulp

2.6. Isolation of an antimicrobial constituent (KVNK/1) by column chromatography

The fraction number F4, eluted in petroleum ether containing 10% acetone, and F5, eluted in petroleum ether containing 30% acetone, exhibited a major spot **[Table– 2]** with the same Rf value of 0.75 **[Figure–3]** and were pooled together. These fractions had also shown strong antimicrobial activity with zone of inhibition more than 15mm using the disc diffusion method against *Salmonella typhi* M531 and S. typhi B330.The solvent from the combined mixture of these two active fractions was evaporated in a rotary evaporator at reduced pressure to obtain cream colored powdery residue. The residue was washed with different solvents to remove the adhering pigmentary material. It was then crystallized from a 3:1 v/v mixture of chloroform and hexane to obtain white colored needles of a pure compound, designated KVNK/1. The compound was characterized using state-of-the-art techniques of spectroscopy and X-ray crystallography [33].

2.7. Phytochemical screening

The methanolic extract and the purified compound were separately tested for the presence or absence of the various groups of phytoconstituents [34, 35].

2.8. Thin layer chromatography [TLC]

The methanolic extracts and the purified compound were subjected to thin-layer chromatography Plates [Kieselgel 60 F254, MERCK] were developed with Choloroform: Acetone: Formic acid [5:4:1] which

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separated the components into a wide range of Rf values. TLC plates were run in duplicate and one set was used as the reference chromatogram. Eluted TLC plates were observed under ambient lighting and illuminated with ultraviolet lamp at 254 nm and 366nm. The other set was used for bioautography.

2.9. *Bio-autography*

After development, the TLC chromatogram plates were dried to remove the solvent. Active extracts were evaluated through the adapted bioautography technique [36, 37]. Briefly, a bacterial suspension [24 hr old culture *Salmonella typhi* M531 & B330] was centrifuged at 3000rpm/min for 20 min. Bacteria were suspended in fresh Muller Hinton [0.6%] agar and the count adjusted to109 bacteria/ml. A fine layer [15 ml] of bacterial suspension was overlaid on the freshly run TLC plates [reference plate, extract and purified compound respectively]. The plates were incubated at 37C for 48h, in humid conditions [petri plates saturated with wet filter paper] and then sprayed with an aqueous solution of 2 mg/ml 2,3,5, - triphenyltetrazolium chloride [TTC, Sigma]. The plates were reincubated at 37C for 24 hours. The areas of inhibition were compared with the Rf value of the related spots on the reference TLC plate.

[III] RESULTS

3.1. *Phytochemical screening*

A variety of phytoconstituents were observed in the fruit pulp. However, the active fraction contained only furanocoumarins [Table– 1].

3.2. *Fingerprint profile and bioautography of the methanolic extract of A. marmelos*

To obtain some information on the active components, plant fractions were analyzed by TLC on silica gel plates. The best solvent system out of all those tested, was chloroform : acetone : formic acid [5:4:1]. Besides this, n-hexane : dichloromethane : methanol [10:10:1.25] also gave good resolution and produced four bands under 366 nm ultraviolet light. The thin layer chomatography of the methanolic extract of *A. marmelos* showed values as given in Table– 2.

Table: 1. Phytochemical screening of *A. marmelos*

S. No	Class of Phytoconstituent	Methanolic extract	Purified fraction
1	Carbohydrates	+	-
2	Alkaloids	+	-
3	Proteins	+	-
4	Amino acids	+	-
5	Triterpenoids	-	-
6	Flavonoids	+	-
7	Tannins	+	-
8	Anthraquinone glycosides	-	-
9	Cardioglycosides	-	-
10	Saponins	+	-
11	Furanocoumarins	+	+

Present (+), Absent(-)

Table: 2. TLC fingerprint profile of methanolic extract of *Aegle marmelos*

Band no.	Rf value
A	0.083
B	0.167
C	0.57
D	0.61
E	0.71
F	0.75

The bioautographs revealed two distinct areas of bacterial growth inhibition in TLC plates [Figure– 2]. The panel A shows the chromatogram of methanolic extract under 366 nm ultraviolet light and the panel B shows the appearance of same chromatogram after treatment with bacterial inoculum, indicating the location of bacterial inhibition zone. These areas corresponded to fractions E [Rf 0.71] and F [Rf 0.75]. Thus, antimicrobial components were present in the methanolic extract of the fruit pulp.

Table: 3. Antimicrobial activity of different fractions of column chromatography [Disc diffusion assay]

S.No.	Fraction code	Eluant	Zone of inhibition [mm]	
			Aegle marmelos [M531]	*Aegle marmelos* [B330]
1	F1	Petroleum ether	_	_
2	F2	Petroleum ether	_	_
3	F3	Petroleum ether : acetone [90 :10]	_	_
4	F4	Petroleum ether : acetone [90 :10]	+++	+++
5	F5	Petroleum ether : acetone [70 : 30]	+++	+++
6	F6	Petroleum ether : acetone [70 : 30]	_	_
7	F7	Petroleum ether : acetone [50 : 50]	_	_
8	F8	Petroleum ether : acetone [50 : 50]	_	_
9	F9	Acetone	_	_
10	F10	Acetone	_	_

(+++) ≥ 15mm zone of inhibition; (-) No inhibition

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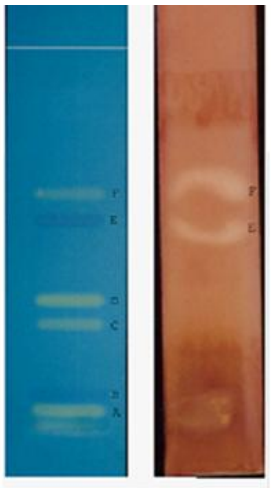

Fig: 2. A Fig: 2. B

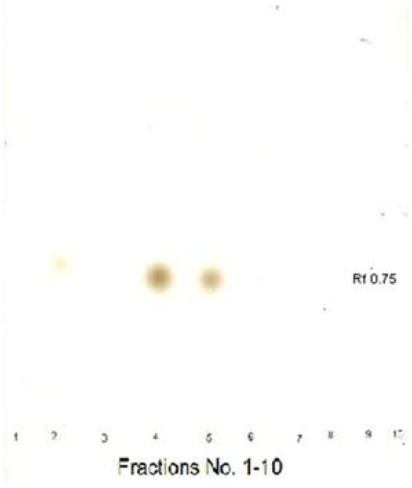

Fig: 3

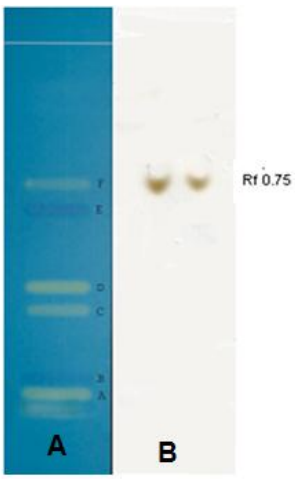

Fig: 4

Fig: 2. TLC and Bioautography of methanolic extract of *Aegle marmelos* against *S. typhi* B330. A) TLC chomatogram of *Aegle marmelos* extract under 366 nm ultraviolet light. **B)** Bioautography of methanolic extract of *Aegle marmelos* against *S. typhi* B330. **Fig: 3. TLC details of fractions 1-10 of methanolic extract of *A. marmelos*, Fig: 4. TLC fingerprint profile of the purified compound [KVNK/1] in comparison to the original extract. A)** Crude extract of *A. marmelos*; **B)** KVNK/1

3.3. *Fingerprint profile and bioautography of an antimicrobial constituent KVNK/1 from fractions of column chromatography*

The fraction number F4, eluted in petroleum ether containing 10% acetone, and F5, eluted in petroleum ether containing 30% acetone, exhibited a major spot **[Figure– 3]** with the same Rf value of 0.75 **[Figure– 3]** and were pooled together. These fractions had also shown strong antimicrobial activity with zone of inhibition ≥ 15mm in disc diffusion method against *Salmonella typhi* M531 and *S.typhi* B330 **[Table–3]**. After further purification by crystallization of the compound, it showed up at the same position [Rf value 0.75] in comparison with the original methanolic extract **[Figure– 4]**.

[V] DISCUSSION

Many drugs are being used as chemotherapeutic agents against various forms of gastrointestinal infections in the children. However, most of the antibiotics invariably have cell toxicity and resistance to the causative agents of these infections i.e. enteric organisms. In recent years, many natural compounds derived from plants or crude plant extracts have proven to exhibit a protective and therapeutic effect in a variety of ailments [38-40]. Plants have a long history of use in the treatment of gastrointestinal disorders [41]. The need to find a safe and highly effective cure for gastrointestinal disorder in children remains a major challenge for modern medicine. Flavones, isoflanones, catechins and tannins present in many plants have been shown to possess anti diarrhoeal potential. Further, some of the herbal medicines and their constituents have been reported to inhibit the ever-increasing number of resistant strains of *Salmonella typhi* [42]. Therefore, an attempt has been made to evaluate the antimicrobial activity of the fruit extract of *Aegle marmelos* which is commonly used in the Ayurvedic system of medicine for a variety of ailments.

Although, a lot of phytochemicals from this plant have been isolated, but to our knowledge, those responsible for antimicrobial activity have not been identified and characterized so far. Therefore this extract was analyzed to identify the bioactive compounds and to examine the future therapeutic potential of the isolated purified compound against clinical pathogenic strains. Our phytochemical analysis ascertained the presence of some potential phytochemical groups i.e., alkaloids, saponins, tannins, flavonoids and furanocoumarins. However, to our knowledge, the antimicrobial effect of coumarins of *A. marmelos* has not been reported so far. In the present phytochemical study, the purified compound KVNK/1 was found to be a furanocoumarin **[Table–1]**. Thus, in consistence with the previous reports that tannins, essential oils and saponins are responsible for antimicrobial activity, the present report has extended the list to furanocoumarins.

In our previous studies, we evaluated the activity of the aqueous and methanolic extract of the fruit pulp of *A.marmelos* against *S. typhi* by using the microdilution techniques [32]. Hence, the methanolic extract was used for further studies. The bioactivity of identified extracts was confirmed in the second stage and quantified by the microdilution and the bioautography assay.

Among the numerous in vitro methods for studying the antimicrobial activity of plant extracts, bioautography has found wide spread application, especially for the detection of new compounds in complex plant extracts. [43]. In this study,

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bioautography revealed promising antimicrobials in the extract of *Aegle marmelos*. These results confirmed the activity of methanolic extract of the fruit pulp of this plant detected in the disc diffusion assay **[Figure–2]**. These antimicrobials were highly active against *Salmonella typhi* B330 and M531.

In some earlier studies done, using the bioautography technique, the phenols and flavonoids were indicated as major active phytochemicals against methicillin Resistant Staphylococcus aureus [44]. Similarly, sterols and terpenes were isolated as antimicrobial components from the methanolic extract of *Eremophila duttonii* [45]. Volatile constituents of *Tambourissa eptophylla* [46] and alkaloids of *Bocconia arborea* have also shown antimicrobial activity against Gram negative and Gram positive bacteria as well as *Candida albicans* [47] using bioautography.

Antimicrobial activity has been shown in the leaves of *A.marmelos* [48]. Our study is in consistence with Raja et al [20] who studied the differential expression of ompC and ompF in multi-drug resistant *Shigella dysentriae* and *Shigella flexneri*. According to these authors, the aqueous extract of *Aegle marmelos* [AEAM] influenced susceptibility of beta lactam resistant Shigella towards beta lactam antibiotics by altering porin channels. They also suggested that AEAM along with beta lactam can be used for the treatment of multi-drug resistant Shigella. Similarly, Shirazi et al [49] studied the antienteric properties of *Salvia officinalis* [sage] extract. The tested extracts [0.1 - 0.05 g/l] exhibited the same effect as ampicillin and streptomycin against *S. typhi*. In another report, Yismaw et al [50] tested the in vitro antimicrobial activity of papaya seed extract against clinical isolates from wound, urine and stool samples and found it be effective with MIC value of 11.8 mg/ml against *S. typhi*.

In the present work, the antimicrobial properties of *A.marmelos* suggested its potential use in traditional med-icine for the treatment of gastrointestinal disorders caused by enteric organisms. As the extract showed very strong anti Salmonella activity, further studies were carried out to purify the antimicrobial compound/s and to characterize them.

Systematic downstream chromatographic analysis of the active methanol fraction done in our lab led to the isolation of white needle shaped crystalline compound [KVNK/1] identified as imperatorin through chemical characterization and spectral studies [33]. In the present work, the antimicrobial properties of *A.marmelos* suggest its potential usage in traditional medicine for the treatment of gastrointestinal disorders caused by enteric organisms. Further studies are being done in the laboratory to elucidate the mechanism of action of these compounds. In conclusion, bioautography proved to be an efficient method in extracting the active compounds.

[VI] CONCLUSION

Aegle marmelos has a great potential in terms of its numerous biological properties. We hereby present a reliable method which can be used to determine the efficacy of *Aegle marmelos* and other medicinal plants in producing antimicrobial substances effective against the clinical enteric pathogen *Salmonella typhi.*

FINANCIAL DISCLOSURE

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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CASE REPORT | OPEN ACCESS

SALVAGING FRACTURED MAXILLARY INCISORS BY REATTACHMENT: CASE REPORTS

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ABSTRACT

Background: *Dental trauma most commonly results in anterior crown fractures. Salvaging such fractured teeth is often a clinical challenge. The development of adhesive dentistry has allowed dentists to reattach the broken segment of the fractured tooth. The reattachment technique offers many advantages such as achieving esthetics, less time consuming etc.* ***Aim:*** *To evaluate different techniques of reattachment, as a treatment modality for fractured maxillary incisors.* ***Case description:*** *The first case report presents a 21 years old male patient with fractured maxillary right central incisor. The treatment carried out included root canal treatment, electrocautery to expose the palatal margin of the root and reattachment of the fractured segment, using resin luting cement. The second case report presents a 24 years old male patient with fractured maxillary right central incisor. Similar treatment as in the first case was followed, with the addition of a fiber-post placement in the root canal before the reattachment of the fragment. At recall visit after 12 months, a stable reattachment was observed in both the cases with good esthetics and periodontal health.* ***Discussion:*** *Treatment of fractured anterior teeth is challenging. Continuous research in adhesive materials has led to the development of innovative techniques like reattachment of fractured segments that offer advantages over routine restorative procedures like composite build-ups, onlays and crowns. The techniques described for the two cases here allow successful reattachment of the broken segments to the fractured teeth, achieving desirable esthetic results. Conclusion: Reattachment resulted in a successful outcome, giving good esthetics and function at a comparatively low cost to the patient.* ***Clinical significance:*** *Reattachment of a tooth fragment is a viable technique that restores function and esthetics, with a very conservative approach.*

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KEY WORDS

Dental trauma; Crown fractures; Fiber-post; Resin luting cement; Fragment reattachment

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[I] INTRODUCTION

The treatment of complicated crown-root fractures in many cases is compromised by fracture lines that are well below the gingival margin or crestal bone. After root canal obturation, proper isolation for a dry operative field is critical to the successful restoration of traumatized teeth. In this respect, a wide range of treatment options have been advocated for fractured permanent teeth including; orthodontic extrusion [1]; osteotomy/osteoplasty [2]; intentional replantation [3]; re-attachment of fragments [4] and the last option being, extraction if nothing else is possible. Re-attachment of a tooth fragment should be preferable to restoring fractured teeth. Besides being a more conservative procedure, there are several advantages to this, such as obtaining esthetics in a single appointment, obtaining a healthy periodontal attachment and maintaining the original tooth contour and translucence [5,6,7]. The present case reports describe the re-attachment of the original fractured tooth fragments with resin luting cement. In one of the cases a glass fiber post, was also used.

[II] CASE DESCRIPTION

2.1. Case 1

A 21 year old male patient was referred to the department of Endodontics, with the complaint of a fractured maxillary right central incisor due to a fall on the sportsfield. He complained of pain during mouth closure and bleeding from the gums at the fracture site. Patient's medical history was non-contributory. Extra-oral examination showed no significant abnormality. Intra-oral examination revealed crown fracture of 11, classified as an unfavorable (plane of the fracture angle extended cervically in a labial to lingual direction with no lingual support to the applied forces) [8]; complicated crown fracture (oblique fracture with fracture line extending subgingivally on the palatal aspect, but without involving the biological width). The fractured coronal segment was highly mobile. Neither laceration nor alveolar bone fracture was evident. Patient had mild pain during examination.

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Radiographic examination (I.O.P.A.) revealed complete root formation and no extrusion of the root was seen **[Figure-1A]**. After taking the patient's consent, it was decided to reattach the broken fragment.

Treatment- step by step procedure

After administration of anesthesia the mobile fractured segment was separated from the gingival tissue attachment **[Figure1B and 1C]**. The pulp chamber of the fractured segment was cleaned and the segment was stored in saline. To expose the fractured margin of the root, the free gingival margin was selectively trimmed on the palatal side by electro cauterization **[Figure- 1D]**. Endodontic treatment of the tooth was performed. Canal was obturated with gutta-percha and AH plus sealer **[Figure- 1E]**.

Acid-etching of the fractured segment and the remaining tooth structure was done. The fractured segment was reattached using the selected shade of composite resin **[Figure- 1F]**. After re-attaching the fragment, a superficial preparation was made on the labial surface extending about 2.5 mm coronally and apically from the fracture line. This was then veneered with a thin composite layer. This technique is useful when the fracture line is still evident after reattachment [8]. After final finishing and polishing the occlusion was evaluated. The patient was informed about the limitations of the technique and was asked to maintain regular follow-up visits.

On recall visit after a month, clinical examination revealed complete healing of the palatal gingival surgical wound and upon recall after 12 months a stable reattachment was observed, with good esthetics and periodontal health **[Figure-1G].**

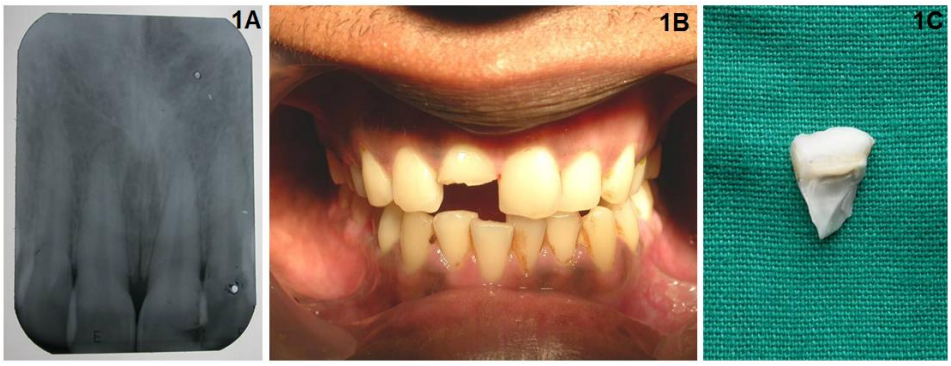

Fig: 1. A) Pre-operative radiograph, **B)** Pre-operative Photograph after removal of fractured segment, **C)** Fractured segment

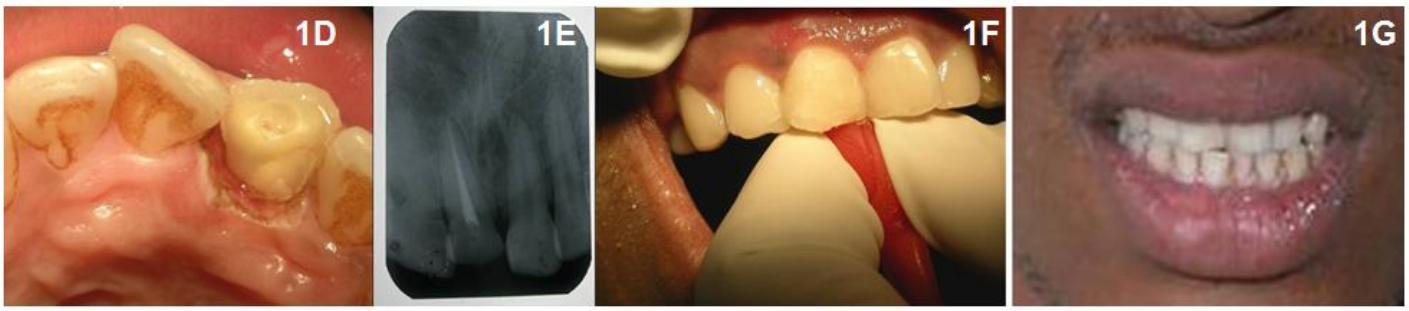

Fig: 1. D) Electrocautery done, **E)** Post-obturation radiograph, **F)** Reattachment, **G)** 1 year follow-up photograph

2.2. Case 2

A 24 year old male patient was referred to the department of Endodontics, with the complaint of a fractured maxillary right central incisor due to trauma, while working on a farm. He complained of pain during mouth closure and bleeding from the gums at the fracture site. Patient's medical history was non-contributory. Extra-oral examination showed laceration of the lower lip. Intra-oral examination revealed crown fracture of 11, classified as a favorable (plane of the fracture angle extended cervically in a lingual to labial direction with maximum lingual support to applied forces) [8]; complicated oblique crown fracture. The fractured coronal segment was mobile **[Figure-2A, 2B]**. Patient had mild pain during examination.

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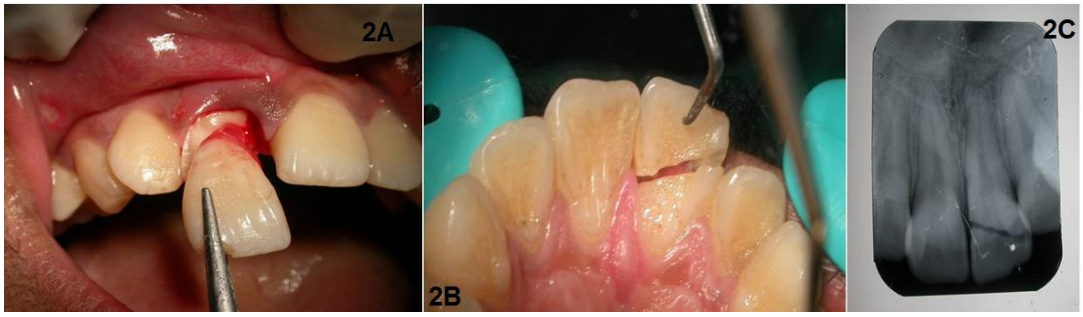

Fig: 2. A) Preoperative labial view, **B)** Preoperative palatal view, **C)** Preoperative radiograph

Radiographic examination (I.O.P.A.) revealed complete root formation and coronal fracture but no extrusion of the root was seen **[Figure- 2C]** Reattachment as an option was discussed with the patient and his consent was obtained.

Treatment- step by step procedure

After anesthesia, the fractured coronal segment was removed **[Figure 2D]** The pulp chamber of the fractured segment was cleaned and the segment was stored in saline. Root canal treatment for the radicular portion was performed. Canal was obturated with gutta-percha and AH plus sealer **[Figure 2E]**. To expose the fractured labial margin of the root, the free gingiva was selectively trimmed by electro cauterization.

Post-space was prepared upto 14mm length and a light transmitting post (DT post) was cemented with Panavia F (Kuraray Dental) dual cure resin cement **[Figure 2F]**. The pulp chamber of the fractured crown segment was prepared to accommodate the head of the post. Acid-etching of the fractured segment, fiber-post and the remaining tooth structure was done. Reattachment was done with Panavia F (Kuraray Dental) as per the manufacturer's instructions **[Figures- 2G and 2H]**. The excess resin was removed gently with a polishing stone. When reattachment was completed, the occlusion was checked to avoid any premature or heavy occlusal contact at the junction of reattachment. In this case, a glass fiber post was used to retain the coronal segment and reduce the stress on the luting material. The post interlocks the two separate fragments and minimizes the stress on the remaining tooth structure [9].

At the 1 month recall examination, the fragment was absolutely firm and upon recall after 12 months a stable reattachment was observed, with good esthetics and periodontal health **[Figure- 2I]**.

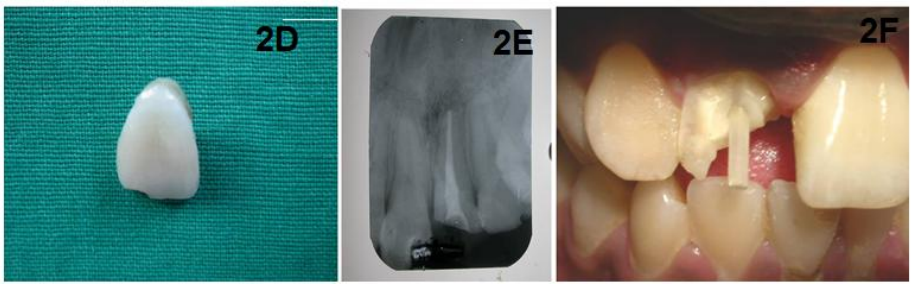

Fig: 2. D) Fractured segment, **E)** Post obturation radiograpgh, **F)** DT post cementation

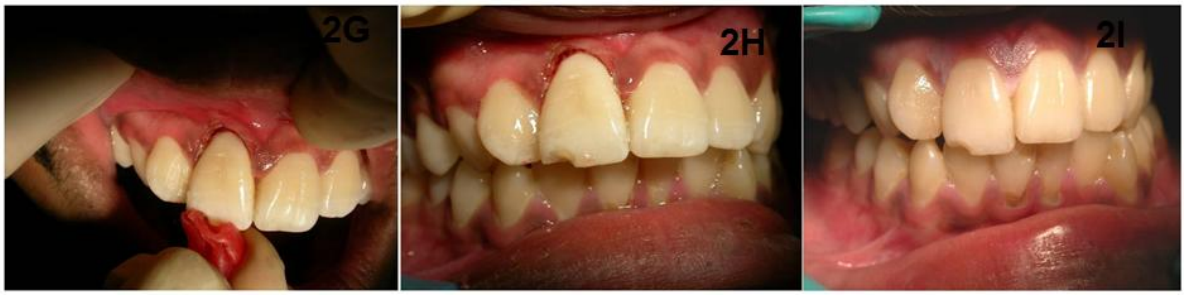

Fig: 2. E) Fragment reattached, **F)** Postpoerative photograph, **G)** 1 year follow-up photograph

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[III] DISCUSSION

The present case reports describe that the reattachment of tooth fragments is an alternative to composite resin build-up, for restoring esthetics and function of fractured teeth.

Anterior crown fractures are a common form of injury that mainly affects children and adolescents. Abundant literature is available on dental trauma, crown fractures and the treatment modalities for such cases. In the pre-adhesive era fractured teeth needed to be restored with either pin-retained inlays or cast restorations that sacrificed healthy tooth structure. Achieving aesthetic requirements were also a challenge for the clinician. The development of adhesive dentistry has allowed dentists to use the broken fragment to restore the fractured tooth [8].

Till date, a lot of different approaches were proposed for the treatment of fractured teeth depending on the location of the fracture [9]. One of the options for managing coronal tooth fractures is the reattachment of the dental fragment [4]. This treatment may offer several advantages over conventional acid-etch composite restoration. Improved esthetics is obtained since the enamel's original shape, color; brightness and surface texture are maintained. In addition, the incisal edge will wear at a similar rate to adjacent teeth, whereas a composite restoration will likely wear more rapidly. Furthermore, this technique can be less time-consuming and provide more predictable long-term appearance [7]. Different reattachment techniques involved are Enamel Beveling; V-shaped Internal Enamel Groove; Internal Dentin Groove; External Chamfer; Over-contour; Simple reattachment etc [8].

Esthetic, biologic and restorative problems may occur as a result of the fracture extending subgingivally and impinging on the biologic width. The treatment options depend on the relationship of the fracture to the alveolar crest, degree of pulpal involvement, amount of eruption, apex formation and esthetic requirement of the patient. Treatment alternatives include crown lengthening to restore the biologic width, flap surgery and ostectomy/osteoplasty to restore biologic width, rapid orthodontic extrusion possibly in conjunction with fiberotomy followed by crown reattachment [9].

[IV] CONCLUSION

Reattachment of a tooth fragment is a viable technique that restores function and esthetics with a very conservative approach. Adhesive techniques, sometimes in conjunction with intra-canal retention, like a post, can be used to reattach fractured segments and an esthetic result can be obtained, with minimal procedure and cost to the patient.

[V] CLINICAL SIGNIFICANCE

Re-attachment of fractured anterior teeth offers an alternative to composite build-ups and more invasive and time consuming treatment options like orthodontic/surgical extrusion followed by crowns. The reattachment technique is less time consuming; not very expensive and fulfills the patient's expectations as far as esthetics is concerned.

FINANCIAL DISCLOSURE

These procedures were carried out, in our institution, free of cost to the patient and we are not supported by any financial assistance.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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EFFECT OF SUPPLEMENTATION OF ZINC: COPPER WITH OR WITHOUT PHYTASE ON BODYWEIGHT GAIN, AVERAGE DAILY WEIGHT GAIN AND FEED CONVERSION EFFICIENCY OF WEANLING PIGLET

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ABSTRACT

The present experiment was conducted to study the growth performances of weanling piglets following supplementation of Zinc:Copper with or without phytase for a period of 4 months. A total of 30 numbers of crossbred piglets (Hampshire X Meghalaya local), aged 60 days, maintained under standard feeding and managemental condition were randomly divided into six groups viz. Group A1, A2,B1, B2, C1and C2 allocating 5 (n=5) piglets comprising 3 males and 2 females in each group. The supplementation was done as follows Group A1= 100ppm Zn + 10ppm Cu, Group A2 =100ppm Zn + 10ppm Cu + 500U phytase, Group B1=200ppm Zn + 20ppm Cu, Group B2 =200ppm Zn +20ppm Cu + 500U phytase, Group C1 =300ppm Zn + 30ppm Cu and Group C2 =300ppm Zn +30ppm Cu + 500Uphytase. The higher BWG (P<0.01) and average daily body weight gain (ADG) were recorded in group C2 (56.90 ± 0.332 kg; 0.486 ± 0.062 kg) followed by C1, B2, B1, A2 and lowest in group A1 (45.00 ± 0.873; 0.373 ± 0.026). Addition of phytase further increased in body weight gain and ADG in group A2, B2, and C2. The best Feed Conversion Efficiency (FCE) was recorded in C2 (2.593 ± 0.037) group followed by C1 (2.773 ± 0.039), B2 (2.839 ± 0.046), B1 (2.971 ± 0.080), A2 (3.046 ± 0.036) and A1 (3.498 ± 0.094).

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KEY WORDS

Zinc; Copper; Phytase; BWG; AWG; FCE; Pig

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[I] INTRODUCTION

As zinc regulates 1000 metallo-enzymes [1] and copper hold the second berth [2] so, they are essential for various biochemical processes of the body. Although, individually both Zn and Cu are essential, for growth, reproduction and better health coverage of livestock. However, the ratio of the two minerals is known to be more important for optimizing the productive and reproductive performances. Under traditional pig management practices, Zinc deficiency is more likely to occur because the pig grow at a rapid rate and reproduces at an early age with larger litter size and shorter inter furrowing interval. Most of the swine feed are plant based containing high phytic acid [3] which hampers the bio-availability of many minerals required for various physiological processes. Therefore, supplementation of phytase offers intensifying opportunity for bioavailability of minerals like phosphorus, calcium, magnesium, manganese, zinc, copper and iron from the dietary source. Therefore, the present experiment was designed to establish a suitable dose level of Zn: Cu with or without phytase for optimizing productive performances in the weaning piglets particularly of North Eastern Region (NER).

[II] MATERIALS AND METHODS

The animal experimentation was conducted at ICAR Research Complex for North Eastern Hill Region, Umiam, Barapani, Meghalaya **[Figure–1]** and the analytical works were done in the Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati -781 022. The pig farm, where the animals were maintain is located at 25º 41'21"N latitude and 91º 55'25"E longitude at an altitude of 1010msl. The agro climatic zone classified for the place is within the subtropical hill agro ecological zone. The maximum and minimum temperature normally ranges from 20.9 to 27.4º C and from 6.7 to 18.1ºC, respectively. The mean annual rainfall is 2399.8 mm with relative humidity between 85 percent and 59 percent. A total of 30 numbers of weaned piglets (10.60 ± Kg body weight; 60 days of age) were used to study the supplemental effect of of Zinc: Copper with and without phytase. The supplementation was done as follows Group A_1= 100ppm Zn + 10ppm Cu, Group A_2 =100ppm Zn + 10ppm Cu + 500U phytase, Group B_1=200ppm Zn + 20ppm Cu, Group B_2 =200ppm Zn +20ppm Cu + 500U phytase, Group C_1 =300ppm Zn + 30ppm Cu

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and Group C_2 =300ppm Zn +30ppm Cu + 500Uphytase. Body weights of the experimental piglets were recorded from 2 months of age at fifteen days interval till attainment of 6 months of age before feeding between 8-10 AM. The average body weight gain (ADG) and feed conversion efficiency (FCE) were calculated. The generated data were statistically analyzed by using SPSS software version 11.5.

Fig: 1. Piglets of one experimental group and measurement of body weigh during experiment.

[III] RESULTS

3.1. Body weight gain

The BWG of the different treatment groups at fifteen days interval from 2 to 6 months of age are presented in **Table–1.** The initial BWG (Mean ± SE) at 2 months of age in A_1, A_2, B_1, B_2, C_1 and C_2 group were recorded as 10.60 ± 0.292, 10.60 ± 0.292, 10.70 ± 0.860, 10.70 ± 0.300, 10.70 ± 0.374 and 10.60 ± 0.400 kg respectively. The final BWG at 6 months of age were recorded as 45.00 ± 0.873, 50.00 ± 0.548, 51.20 ± 1.158, 53.00 ± 0.612, 54.00 ± 0.500 and 56.90 ± 0.332 kg for A_1, A_2, B_1, B_2, C_1 and C_2 groups respectively. The highest BWG at 6 months of age was recorded in C_2 group followed by C_1, B_2, B_1, A_2 and lowest in A_1 group. As the supplemental dose of Zn: Cu increased, the BWG also recorded higher values accordingly. Further, phytase supplementation showed a positive increment in BWG in A_2, B_2 and C_2 groups when compared with corresponding non supplemented groups i.e. A_1, B_1 and C_1. A definite linear trend of increase in BWG was recorded throughout the experimental period, irrespective of the treatment groups. The Critical Difference Test at 6 months of age showed that the BWG was significantly ($P<0.05$) highest in C_2 group when compared to that of other groups (C_1, B_2, B_1, A_2 and A_1). There was no significant difference in BWG between A_2 and B_1 groups and B_2 and C_1 groups

3.2. Average daily weight gain

The ADG of the different treatment groups recorded at fifteen days interval from 2 to 6 months of age are presented in **Figure–2.** The ADG (Mean ± SE) in A_1, A_2, B_1, B_2, C_1 and C_2 groups at 2.5 months of age were recorded as 0.173 ± 0.012, 0.253 ± 0.027, 0.252 ± 0.063, 0.273 ± 0.019, 0.279 ± 0.027 and 0.306 ± 0.034 kg respectively. At 6 months of age the ADG for the above treatment groups were 0.373 ± 0.026, 0.399 ± 0.047, 0.419 ± 0.055, 0.419 ± 0.030, 0.459 ± 0.046 and 0.486 ± 0.062 kg respectively. The highest ADG at 6 months of age was recorded in C_2 group followed by C_1, B_2, B_1, A_2 and lowest in A_1 group. Supplementation of Zn: Cu at higher concentration resulted higher ADG in all the treatment groups. Additional phytase supplementation in A_2, B_2 and C_2 groups resulted higher ADG when compared with corresponding phytase non supplemented groups i.e. A_1, B_1 and C_1. In all the treatment groups the ADG was found to be increased along with advancement of treatment period. Although, the ADG at 6 months of age showed an apparent variation among the different treatment groups the Critical Difference Test at 6 months of age revealed no significant difference between the groups.

3.3. Feed conversion ratio

The FCE of the different treatment groups are presented in **Figure–3**. The values of the FCE (Mean ± SE) were recorded as 3.498 ± 0.094, 3.046 ± 0.036, 2.971 ± 0.080, 2.839 ± 0.046, 2.773 ± 0.039 and 2.593 ± 0.037 in A_1, A_2, B_1, B_2, C_1 and C_2 groups respectively. In the present experiment, the FCE was found to be dependent on the dietary level of Zn:Cu and phytase. The best FCE was recorded in C_2 group followed by C_1, B_2, B_1, A_2 and A_1 groups**.** At 6 months of age showed that the FCE was significantly ($P<0.05$) highest in C_2 group. There was no significant difference in FCE between A_2 and B_1 groups and B_2 and C_1 groups. The C_2 group recorded the best FCE.

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Table-1: Body weight (kg, Mean ± SE) in different treatment groups at fifteen days interval from 2 to 6 months of age

Treatment group	Total body weight gain (kg)								
	Age (months)								
	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
A_1	10.60^{a} ± 0.292	13.20^{a} ± 0.255	16.60^{a} ± 0.292	20.90^{a} ± 0.458	25.30^{a} ± 0.663	29.90^{a} ± 0.332	34.50^{a} ± 0.500	39.40^{a} ± 0.678	45.00^{a} ± 0.837
A_2	10.60^{a} ± 0.292	14.40^{b} ± 0.400	18.80^{b} ± 0.374	23.40^{b} ± 0.430	28.20^{b} ± 0.604	33.20^{b} ± 0.374	38.50^{b} ± 0.500	44.00^{b} ± 0.548	50.00^{b} ± 0.548
B_1	10.70^{a} ± 0.860	14.40^{b} ± 0.400	18.90^{b} ± 0.458	23.60^{b} ± 0.510	28.40^{bc} ± 0.400	34.20^{b} ± 0.583	39.80^{bc} ± 0.860	44.90^{b} ± 0.510	51.20^{b} ± 1.158
B_2	10.70^{a} ± 0.300	14.80^{b} ± 0.339	19.40^{bc} ± 0.485	24.20^{bc} ± 0.604	29.40^{cd} ± 0.245	34.70^{c} ± 0.255	40.50^{c} ± 0.758	46.70^{c} ± 0.374	53.00^{c} ± 0.612
C_1	10.70^{a} ± 0.374	14.90^{b} ± 0.332	19.60^{bc} ± 0.292	24.80^{bc} ± 0.374	29.80^{d} ± 0.255	35.20^{c} ± 0.464	41.00^{c} ± 0.612	46.90^{c} ± 0.430	54.00^{c} ± 0.500
C_2	10.60^{a} ± 0.400	15.20^{b} ± 0.374	20.00^{c} ± 0.316	25.20^{c} ± 0.374	31.00^{e} ± 0.447	37.20^{d} ± 0.604	43.60^{d} ± 0.510	49.60^{d} ± 0.696	56.90^{d} ± 0.332

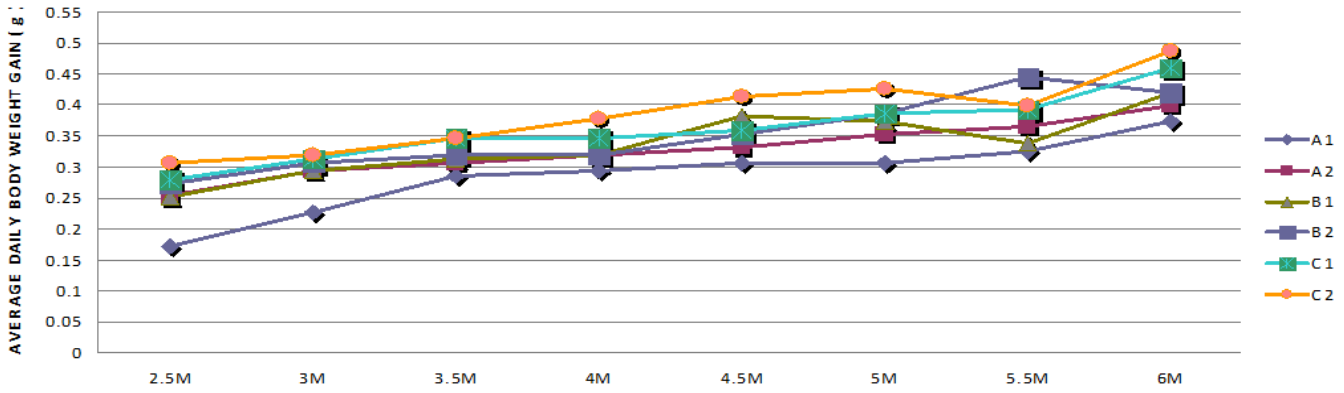

Fig: 2. Average daily body weight gain in different treatment groups at fifteen days interval from 2.5 to 6 months of age

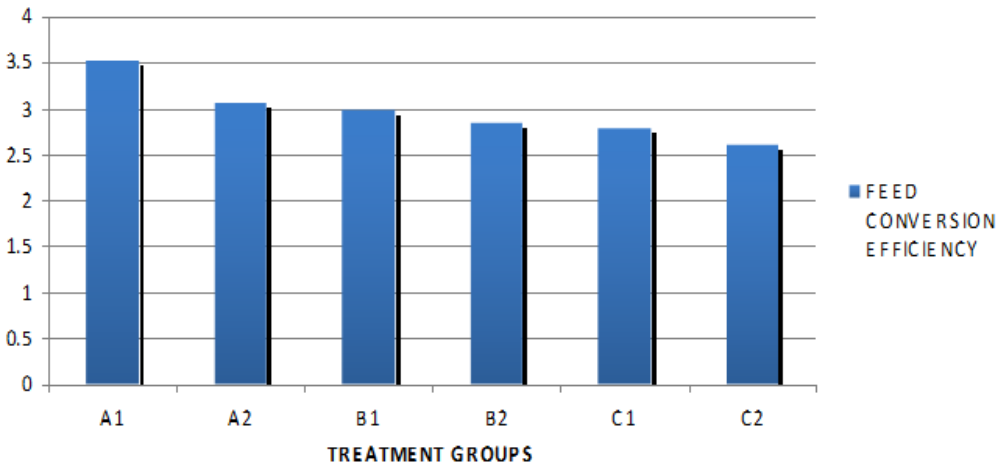

Fig: 3. Feed conversion efficiency in different treatment groups for the whole experimental period (4 months) from 2 to 6 months of age

[IV] DISCUSSION

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The initial BWG and ADG (Mean ± SE) recorded from 2 months of age followed by fifteen days interval until 6 months of age in the experimental animals of group A1, A2, B1, B2, C1 and C2 are presented in the **Table– 1** and **Figure–2.** Irrespective of the treatment group, increase in BWG and ADG was recorded with advancement of age. This indicates that all the experimental animals were maintained properly in respect of feeding, management and health coverage. However, higher BWG and ADG was recorded when the supplemental dietary level of zinc (Zn) :copper (Cu) was increased from 100:10ppm to 300:30ppm. Further increased in BWG and ADG was observed when phytase was added along with the higher supplemental level of Zn:Cu in group A2, B2, and C2. At 6 months of age, highest BWG (56.90 ± 0.332 kg) and ADG (0.459 ± 0.046) was recorded in group C2 and lowest BWG (45.00 ± 0.873 kg) and ADG (0.373 ± 0.026) was observed in group A1.

In the present experiment, A1 and A2 groups received the supplemental Zn as per NRC (1998) requirement. Rest of the experimental animal received higher than NRC (1998) recommendation. Many workers [3-5] opined that dietary requirement of Zn in porcine might be higher than the recommended NRC (1998) level i.e., 80-100ppm in different growing phases. Results of the present study clearly indicate that dietary level of Zn should be more than 100ppm to enhance the growth performances of weaned piglets. Borah (2009) [6] also reported that supplementation of 500 ppm Zn in pig diet resulted better weight gain. The higher requirement of Zn in NER might be due to presence of some mineral present in the drinking water, which might have reduced the bioavailability of Zn. The significantly higher iron content in soil and water of the NER might have reduced the bioavailability of Zn, resulted in higher demand of supplemental Zn in the diet. Brandao (1990) [7] described the involvement of Zn in growth and development of tissues enhancing perception of taste, regulation of appetite, increased food consumption, DNA and RNA synthesis, cell transcription in the synthesis of somatomedin-C, alkaline phosphatase, collagen, oesteocalcin and participating in protein, lipid and carbohydrate metabolism. It was further, confirmed that, adequate dietary level of Zn is essential for optimizing growth [8], reproduction [9, 10], strengthening the immune system [11, 12] and maintaining general health [8, 13]. Berger (2002) [14] also attributed the beneficial effect of Zn and Cu in promoting growth to the stimulatory affect of gustin and carbonic anhydrase are Zn dependent enzymes which are required for development of the taste buds which may enhance the feed consumption ,their assimilation in the biological system and ultimately causing increase body weight gain . Both Cu and Zn have some antibacterial properties [15] which may also explain the growth promoting effect. However, role of Zn controlling about 1000 enzyme activities [1] may be implicated in growth promoting effect of Zn in pig. Beside antibacterial activity of the Cu it is also essential for hemoglobin synthesis and therefore responsible for continuous supply of O_2 and removal of CO_2 to and from the cells at optimum level.

Present experiment also revealed that additional supplementation of phytase further significantly ($p<0.05$) increased the BWG. Earlier workers [16-18] also demonstrated that supplemental phytase resulted in improvements of BWG and ADG. This might be due to the fact that, supplementation of phytase offers intensifying opportunity for bioavailability of minerals like phosphorus (P), calcium (Ca), magnesium(Mg), manganese (Mn), zinc (Zn), copper (Cu) and iron (Fe) from the dietary source besides protein. The main storage form of P in seed is phytate (the salt of phytic acid) which reduce the P utilization in pig. Under normal physiological condition phytate is a negatively charged ion i.e. able to bind cations like Ca, Mg, Zn, and protein [19, 20]. Supplementation of phytase in the diet hydrolyzes the ortho- phosphate group from phytate more efficiently resulting the liberation of phytate bound nutrients and the net result is the greater bioavailability of P, Ca, Mg, Mn, Zn, Cu, Fe and dietary proteins [21] which might have accerelarated the growth process, participating in various biochemical processes of the body systems.

The FCE (Mean ± SE) was 3.498 ± 0.094, 3.046 ± 0.036, 2.971 ± 0.080, 2.839 ± 0.046, 2.773 ± 0.039 and 2.593 ± 0.037 in A1, A2, B1, B2, C1 and C2 group respectively. The best FCE was recorded in C2 group followed by C1, B2, B1, A2 and A1 group **[Figure-2].** Pig is known as excellent converter of feed when compared to other livestock species. However optimization of FCE depends on the interaction of different macro and micro nutrients. Therefore strategic nutrient intervention is essential for better FCE which was supported by the higher BWG and ADG. Many earlier workers [22-24] have reported that, Zn supplementation with different dose level, age group and periods leads to improve the FCE in pig and was in agreement with the present findings. Present finding is also in agreement with improved FCE when pigs were supplemented with phytase [25-27].

[V] CONCLUSION

The present study established that Zn:Cu plays a significant role in growth and addition of phytase may reduced the supplemental level of Zn:Cu in the feed. Since cost of phytase is higher, rather a higher level of zinc can be incorporated. It was also established that 200ppm of Zinc along with 500U phytase or 300ppm of Zinc in the diet of pig showed higher growth and better FCE. Therefore, to enhanced the growth performances the dietary level of Zn must be higher than 100ppm (NRC recommended) particularly in the agroclimatic zones of Northeastern region in India

FINANCIAL DISCLOSURE

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CONFLICT OF INTERESTS

The authors declare no competing interest in relation to work.

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CYTOTOXIC EFFECTS OF METHANOL EXTRACT OF RAW, COOKED AND FERMENTED SPLIT BEANS OF *CANAVALIA* ON CANCER CELL LINES MCF-7 AND HT-29

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ABSTRACT

In vitro cytotoxicity evaluation of methanol extract of raw, cooked and solid-substrate fermented (Rhizopus oligosporus) split beans of wild legumes (Canavalia cathartica and C. maritima) of coastal sand dunes of the Southwest India was carried out. Cytotoxic activity (ED_{50} and cytotoxicity) of methanol extracts was tested by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Differential impacts on the cancer cell lines MCF–7 and HT–29 were seen even though both plant species grow in the same habitat. Methanol extract of cooked (C. maritima) and fermented (C. cathartica) split beans showed better in vitro anticancer activities compared to the raw beans. It is concluded that active principles of methanol extract of cooked and fermented Canavalia beans have potential to inhibit cancer cell lines MCF-7 and HT-29. Besides, it is possible to use extracts of cooked/fermented beans to control colon cancer by diet management.

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KEY WORDS

Cytotoxicity; Canavalia; Wild legumes; Solid-substrate fermentation; Rhizopus oligosporus; Cancer cell lines

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[I] INTRODUCTION

Out of 7.6 million deaths worldwide, cancer is one of the leading causes for human mortality in developed countries attained second place in developing countries after cardiovascular diseases [1,2]. Lung, stomach, liver, colon and breast cancers are the ailments for death every year. Most of the cancer deaths are caused largely because of aging and increasing adoption of cancer-causing behavioral and dietary risks including smoking, high body mass index (BMI), lack of physical activity and low fruit-vegetable intake [3]. The global burden of cancer is predicted to increase with an estimation of 13.1 million deaths during 2030 [1]. Present cancer treatments by radiation and chemotherapy pose serious side effects like fatigue, diarrhoea, nausea, hair loss, skin problems, malfunction of urinary bladder and decrease in RBCs due to cytotoxicity and genotoxicity of radiation and chemotherapeutic agents on the non-tumor cells [4].

Over the past few years, there has been growing interest in developing plant-based anticancer drugs due to their diverse pharmacological properties and benefits [5]. The use of natural products in anticancer therapy has a long history in folk medicine, which is part and parcel of traditional and allopathic medicines. Many drugs currently used in chemotherapy originated from different plant species or derivatives of a natural prototype. According to Cragg and Newman [6], more than 50% of drugs that undergo clinical trials for anticancer activity are derived from natural sources. Numerous epidemiological studies especially colorectal cancer have clearly showed an inverse relationship between the diet rich in vegetables/legumes and incidence of cancers [7]. The plant-derived anticancer drugs act via different pathways, which ultimately result in activation of apoptosis of cancer cells leading to cytotoxicity [8]. Legume grains play a major role in the fulfillment of diets of human beings throughout the world. Supplementation of legumes in the diets is reported to be one of the promising approaches (diet-management) to combat various free radical-mediated chronic diseases [9, 10].

The nutritional potency of underutilized legumes, *Canavalia cathartica* and *Canavalia maritima* of the coastal sand dunes (CSD) of Southwest coast of India has been reported by many researchers [11-14]. Xu et al. [15] have reported that pterocarpin derivative [(−)-mediacarpin] extracted from *C. maritima* inhibits the growth of HeLa cells *in vitro* by inducing apoptosis. Lectins derived from *Canavalia ensiformis* (ConA) and *Canavalia brasiliensis* (ConBr) showed anti-proliferative effects in human leukaemia cell lines (MOLT-4 and HL-60) [16]. Animal studies have suggested that L-canavanine present in *Canavalia* spp.

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possess potent antineoplastic activity, which can be used in treating pancreatic cancer [17, 18]. To consider *Canavalia* beans as potential nutraceutical agent, its effect on cancer lines are essential. Therefore, the current study reports differential cytotoxic effects of raw, cooked and solid-substrate fermented (SSF) (*Rhizopus oligosporus*) split beans of CSD *Canavalia* on cancer cell lines. As fermented beans showed high bioactive and antioxidant potential [19], it is hypothesised that the fermented beans also exhibit high cytotoxic activity on cancer cell lines.

[II] MATERIALS AND METHODS

2.1. Seed samples and fermentation

Seed samples of *Canavalia cathartica* Thouars and *Canavalia maritima* Thouars were collected from three locations of the coastal sand dunes of Someshwara, Southwest India (12º47′N, 74º52′E) during summer (February–March, 2012). Undamaged seeds were separated from dry pods, sun-dried for two days and dehulled. First set of split beans (25 g) were transferred to conical flask (250 mL), soaked in distilled water (1:3 w/v) followed by pressure cooking (6.5 L, Deluxe stainless steel; *TTK* Prestige™, Prestige Ltd., India). The cooked split beans were spread on aluminium foil, dried in an incubator (45±2°C), milled (Wiley Mill, mesh # 30) and stored in air-tight glass containers. Another set of split beans were pressure-cooked, inoculated with two 5 mm plugs of 3-day-old cultures of *Rhizopus microsporus* var. *oligosporus* (Saito) Schipper and Stalpers (MTCC # 556; strain designation # 22959; Institute of Microbial Type Culture Collection, Chandigarh, India) and allowed for solid-substrate fermentation for 7 days at 37°C. Fermented split beans were spread on aluminium foil, oven dried at 45±2°C, powdered and preserved in air-tight glass containers for analysis.

2.2. Extraction

Samples were extracted in methanol using Soxhlet extractor [20]. Flours of raw, cooked and SSF beans were packed in thimbles, covered with glass wool and extracted with methanol (200 mL) (50-65ºC) in a Soxhlet extractor. The rate of condensation was fixed to 150 drops/min and the extraction was carried up to 7 hr. After recovering the solvent, the extract was concentrated by evaporating the solvent using flash evaporator and stored at –20ºC.

2.3. Cell lines

The cell lines, MCF–7 and HT–29 were procured from National Centre for Cell Sciences (NCCS), Pune, India. The MCF–7 is a human breast adenocarcinoma cell line [21]. It retains the characteristics of differentiated mammary epithelium including estradiol synthesis. This makes the MCF–7 cell line an estrogen receptor positive control cell line [22]. The HT–29 human colon carcinoma cells in culture show similar characteristics of enterocytes and these cells have been used to study intestinal drug, nutrient transport and metabolism [23,24].

2.4. Cytotoxicity assay

The *in vitro* cytotoxic activity of the extracts was tested by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25] with a slight modification. The MCF–7 and HT-29 (5×10^3 cells) in Dulbecco's Minimal Essential Medium (DMEM) (100 µL) with Foetal Bovine Serum (10%) were incubated overnight (37°C, 5% CO2) in a 96 well plate. Methanol extracts of test samples (*Rhizopus oligosporus*, raw, cooked and fermented flours of *C. cathartica* and *C. maritima*) at different concentrations (50, 100, 250 and 500 µg/mL; 1, 2.5, 5 and 10 mg/mL) were added to microtitre plate. Doxorubicin (5 µg/mL), an anticancer drug was used as internal positive control, DMEM served as negative control and the wells without any cells served as blank. It was incubated for 48 hr (37°C, 5% CO2, humidity 80-90%). After initial incubation, MTT (20 µL of 5 mg/mL) in phosphate buffered saline was added to each well and incubated further for 4 hr (37°C, 5% CO_2, humidity 80-90%). The medium together with MTT was aspirated and dimethylsulfoxide (DMSO) (200 µL) was added. The absorbance in each well was measured at 570 nm using micro-titre plate reader. Inhibition of cells (%) was calculated:

Inhibition (%) = 100 – [(Mean OD for test sample / mean OD for the control) × 100]

The concentration at which the sample exhibits 50% of its maximum activity (i.e., effective dosage: ED_{50}) was calculated using the ED_{50} plus v1.0 software.

2.5. Data analysis

The difference between the cytotoxic activity of methanol extracts of raw and processed (cooked and cooked + fermented) beans was assessed by One-way analysis of variance (ANOVA) (SigmaPlot 11; Systat Software Inc., USA).

[III] RESULT AND DISCUSSION

Cytotoxic effects on human cancer cell lines was performed using MTT assay, which was based on the principle of conversion of purple tetrazolium salt into formazan by the methanol extracts of the test samples such as raw, cooked and solid-substrate fermented (*Rhizopus oligosporus*) beans of *C. cathartica* and *C. maritima* [25]. The results demonstrated that methanol extracts of *Canavalia* bean exhibited selective *in vitro* cytotoxic activity towards MCF–7 and HT–29 cell lines. Among the two cancer cell lines tested, viability of HT–29 cells was most affected especially by methanol extracts of cooked *C. maritima* beans followed by fermented *C. cathartica* beans **[Table- 1]**. Similarly, in bean extracts the ED_{50} value was lowest in cooked beans of *C. maritima* and fermented beans of *C. cathartica*. However, the ED_{50} values were the lowest and the cytotoxicity was highest in *R. oligosporus*. The quantity of bioactive compounds (total phenolics, tannin and vitamin C) and antioxidant potential of *C. maritima* fermented with *R. oligosporus* differed from that of *C. cathartica* [19]. Likewise, the *in vitro* cytotoxicity potential was also differed and thus did not correspond to the *in vitro* anti-cancer activity of two *Canavalia* spp. in the present study. On the contrary, the black soybean fermented with *R. oligosporus* exhibited higher phenolics, flavonoids and antioxidant activity, which corresponds to the effective cytotoxic activity against HeLa-S3 and Raji cell lines [26]. Similarly, fermented *R. oligosporus* soymilk showed selective cytotoxic effect on Hep 3B with ED_{50} value of 150.2 µg/mL [27]. It is interesting to note that certain strains of *Rhizopus microsporus* are known to produce rhizoxins, which shows anti-tumor activity (Jennessen et al. [28]. The ED_{50} of methanol extracts of cooked *C. maritima* was lower compared to methanol extracts of raw and fermented beans as well as *C. cathartica* (850-893 vs.1357-4063 µg/mL)

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on both cell lines [Table- 1]. Gazzani et al. [29] predicted that the environmental factors (climatic, growth conditions, ripening stage, temperature and duration of storage) and thermal treatment influence the antioxidant activity. Similarly, the cytotoxic potential might also varied exist in the same coastal sand dunes.

The initial hypothesis proposed (fermented beans show higher cytotoxic activity than the raw and cooked beans) was true only for *C. cathartica*. Future studies should focus on purification of anti-cancer compounds from *Canavalia* beans and their *in vivo* evaluation for therapeutic applications.

Table: 1. Effective dosage (ED_{50}) and cytotoxicity (% inhibition) of methanol extracts of raw, cooked, fermented beans of *C. cathartica* and *C. maritima* on cancer cell lines MCF–7 and HT–29 in culture in comparison with *Rhizopus oligosporus* (n=3; mean±SD) (low ED_{50} and high cytotoxicity are in bold-face)

	ED_{50} value (µg/mL)		Cytotoxicity at 1 mg/mL (% inhibition)	
	MCF–7	HT–29	MCF–7	HT–29
Doxorubicin	–	–	68.59	48.42
Rhizopus oligosporus	390.05±0.97	461.19±1.01	94.21±0.21	89.85±0.61
Canavalia cathartica				
Raw beans	3350.67±0.49^{a}	2844.41±0.03^{a}	NI	25.14±0.06^{a}
Cooked beans	3572.65±0.20^{ac}	4063.85±0.07^{ac}	NI	21.57±0.45^{ac}
Fermented beans	**2049.20±0.24^{ad*}**	**2127.80±0.29^{ad*}**	**14.04±0.16**	**34.85±0.13^{ad*}**
Canavalia maritima				
Raw beans	1408.61±0.74^{a}	1747.71±0.27^{a}	31.90±0.92^{a}	27.14±0.04
Cooked beans	**892.89±0.06^{ac}**	**849.83±0.87^{b*c}**	**63.63±0.69^{ac}**	**60.42±0.43**
Fermented beans	1505.35±0.86^{ad*}	1357.00±2.00^{bc}	26.11±0.03^{ad*}	NI

Different letters across the rows are significantly different (*, P < 0.05); –, Not determined; NI, No inhibition.

[IV] CONCLUSION

The active principles of methanol extract of cooked and fermented *Canavalia cathartica* and *C. maritima* beans of the coastal sand dunes of Southwest coast of India have potential to inhibit cancer cell lines MCF-7 and HT-29. Besides, it is possible to use extracts of cooked and fermented beans to control colon cancer by diet management. Further studies are necessary to assess purified bioactive compounds of *Canavalia* beans to improve the efficacy.

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CONFLICT OF INTERESTS

Authors declare that there are no conflicts of interest

FINANCIAL DISCLOSURE

Nil

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