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In-Vitro Cytotoxic Effect of Fermented and Non-Fermented Plant Extracts Against HCT-116, PC-3 and A-549 Cell Lines

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Abstract: Methanolic and ethyl acetate extracts of *Acacia catechu* (Heartwood), *Terminalia chebula* (Fruits), *Terminalia Bellirica* (Fruits), *Cinnamomum tamala* (Leaves) and *Ocimum sanctum* (Leaves) were used in the present study and these medicinal plants possesses various therapeutic properties. Also, the probiotics used for fermentation processes were *Saccharomyces cerevisiae, Bacillus subtilis, Lactobacillus acidophilus, Lactobacillus plantarum* and *Lactobacillus casei*. In the current investigation, we investigated in-vitro-cytotoxic potential of non-fermented and fermented plant extracts against three different cell lines namely, colorectal carcinoma (HCT-116), prostrate small cell carcinoma (PC-3) and adenocarcinomic human alveolar basal epithelial cells (A-549) by following Sulforhodamine B (SRB) method. The results showed that fermented plant extracts possessed higher IC_{50} value in comparison to their non-fermented extracts. In this investigation, *A. catechu* methanolic extract fermented by *L. acidophilus* showed highest cytotoxic activity with IC_{50} value of 31.96 µg/mL against A-549. Whereas, the lowest cytotoxic activity showed by non-fermented ethyl acetate extract of *T. bellirica* and non-fermented extracts of *C. tamala* and *O. sanctum* with IC_{50} values of 2500 µg/mL. Now, it has been established from the present study that the medicinal plant extracts fermented by probiotics under optimized conditions possesses higher cytotoxic effect in comparison to their non-fermented extracts.

Keywords: *Cytotoxic Activity, Fermentation, Cell Lines, Plant Extracts and Probiotics Etc.*

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I. INTRODUCTION

Pharmaceutical companies and research groups have been using phytochemicals extracted from medicinal plants in the development of medicines to treat various diseases (Wadood A. *et al.,* 2013). Microbial induced fermentation has been used to improve the bioactive properties of natural products since prehistoric times. Fermentation is a biological process that involves a series of biochemical reactions to convert complex organic metabolites into useful end products (Khirwadkar P. *et al.,* 2014; Smitha M.S. *et al.,* 2017). Fermentation process of plant extracts uses microbes as a biocatalyst to modify organic chemical conjugates into optically pure and free forms of chemical derivatives. It involves processes such as oxidation, condensation, hydrogenation, hydroxylation, isomerization, methylation, hydrolysis, reduction, glycosylation and synthesis of new carbon bonds (Smitha M.S. *et al.,* 2017; Nigam S. *et al.,* 2013; Salter R. *et al.,* 2019). Fermented plant extract is a type of plant-based functional food that is widely consumed in China, Japan and other Asian countries. Fermented plant extracts can be made from a variety of plants including

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cereals, legumes, fresh fruits, vegetables and edible fungus. The microbes used in fermentation processes are yeast, lactic acid bacteria and acetic acid bacteria to produce a beverage or other physical forms (Blandino A. *et al.,* 2003). Traditional fermented plant extract products include pickles, bean paste, natto, miso and so on. Other fermented products, such as wine, vinegar and yoghourt are not the same as fermented plant extracts. One reason is that there is no or very little alcohol present (Altay F. *et al.,* 2013). Another advantage is that it is made entirely of plants and edible fungus. One more is explanation that there are more types of microbes involved in the development of fermented plant extracts, but in wine (yeast), vinegar (acetic acid bacteria), and yoghourt (lactic acid bacteria), there is only one main type of microbe, On the other hand in the development of fermented plant extracts one or more microbes are utilized (Urbonaviciene D. *et al*., 2015).

In view of above facts, a research problem was planned with the objective to investigate the potential of probiotic mediated fermentation of plant extracts to enhance cytotoxic potential of fermented plant extracts against three different cell lines.

II. MATERIALS AND METHODS

➢ *Collection of Plant Materials:*

Five different medicinal plants namely, *Acacia catechu* (Heartwood), *Terminalia chebula* (Fruits), *Terminalia bellirica* (Fruits), *Cinnamomum tamala* (Leaves) and *Ocimum sanctum* (Leaves) were collected from Jammu district, Jammu and Kashmir, India. Extracts prepared using two different solvents namely, Methanol and ethyl acetate (Fig. 1 and 2).

➢ *Probiotics used for Fermentation Process*

The probiotic microbes used in the study were *S. cerevisiae* (MTCC-180), *B. subtilis* (MTCC-2389), *L. acidophilus* (NCIM-5307), *L. plantarum* (MTCC-1407) and *L. casei* (NCIM-5304).

➢ *Preparation of Plant Extract*

Fresh plant part of *A. catechu*, *T. chebula*, *T. bellirica*, *C. tamala* and *O. sanctum* were first washed using tap water followed by three times with distilled water and then allowed to dry in shade at room temperature. Shade dried plant parts were grounded to fine powder using a grinder (Model: Twister, Bajaj Electricals Ltd., Mumbai, India). Methanol and ethyl acetate were used as solvents for preparation of plant extracts. The plant extracts were prepared by suspending 100 g of plant part powder in 300 mL of each solvent and placed on rotary shaker at room temperature for 24 h. After 24 h the 28 filtrate was collected in 500 mL of Erlenmeyer flask and the process was repeated three times. The filtrate so obtained from two different solvents was concentrated to dryness by evaporating under reduced pressure at 45 °C using vacuum rotary evaporator and stored at -20 ˚C until further use.

➢ *Preparation of Probiotic Microbial Cultures:*

Cultures of probiotic microbes to be used in fermentation processes were prepared using standard procedures.

- *Revival of Lyophilized Dried Cultures:*

Five lyophilized dried probiotic microbial cultures namely *S. cerevisiae*, *B. subtilis*, *L. acidophilus*, *L. plantarum* and *L. casei* were revived.

For revival of lyophilized probiotic culture the ampoule was sterilized by wiping with cotton wool containing 70% ethanol. Then covered the ampoule with a sterile cotton sheet and made a fine cut at the neck of the ampoule. Using a sterile Pasteur pipette, added 500 µL of suitable rehydration solution (yeast peptone dextrose broth for *S. cerevisae* and nutrient broth for probiotic bacteria) into the ampoule. The culture samples were poured on petriplates containing solidified nutrient agar and incubated under the optimized conditions (incubation for 24 h at 27 °C for *S. cerevisae* and incubation for 24 h at 37 °C for probiotic bacteria).

- *Sub-Culturing of Probiotic Micro-Organisms used in the Study:*

Sub-culturing of probiotic microorganism was performed by transferring a loop full of culture using inoculation wire loop from the mother culture plate to fresh plate containing NA medium followed by incubation of culture in case of probiotic bacteria at 37 °C for 24 h and in case of *S. cerevisae* at 27 °C for 24 h, which were then stored in refrigerator for further study.

➢ *Determination of CFU/mL of Probiotics:*

Colony forming unit per mL (CFU/mL) of probiotic microbes under study, was determined at late log phase by serial dilution method. 100 µL of serially diluted culture was plated on petriplates containing sterilized solidified nutrient agar medium and were incubated for 24 h at 37 ˚C (in case of probiotic bacteria) and at 27 °C (in case of *S. cerevisae*) in B.O.D incubator. After 24 h, petriplates containing 30 to 300 colonies were selected for counting colony forming units (CFU). CFU/mL was calculated using following formula;

CFU/mL = (Number of colonies × dilution factor)/volume of culture plated in mL

Log CFU/mL was used each time for determining inoculum load used for fermentation processes by recording absorbance at 600 nm.

➢ *Optimization of Fermentation Parameters*

Fermentation parameters for carrying out fermentation process of plant extract under study was optimized to assess optimum growth conditions and maximize efficiency of fermentation using shake flask method. Single parameter was optimized at one time while keeping other parameters constant. Optimization was done for inoculum load, pH, temperature and incubation time. Non-inoculated plant extract was maintained as control for comparison.

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➢ *Determination of Cytotoxic Activity:*

Sulforhodamine B (SRB) assay was carried out as described by Skchan *et al.*, 1990 using SRB dye. The non-fermented and fermented plant extracts in different concentration were incubated with three different cell lines namely, HCT-116, PC-3 and A-549 for 48 h. After incubation 50 µL chilled 50% trichloroacetic acid (TCA) was gently layered on top of the medium in all the wells to produce a final concentration of 10% and incubated at 4° C in a refrigerator to fix the cells attached to the bottom of the wells. After 1 h the plates were taken out of the refrigerator and all the content of all the wells were pipette out and supernatant was discarded. The plates were then washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc. For washing the wells of tissue culture plates were filled with distilled water and the liquid in the wells was discarded by sharply flicking plates over a sink. Plates were air dried and stored until use. SRB solution (100 µL was added to each well of the plates and incubated for 30 minutes at room temperature. The unbound SRB was removed quickly (to avoid desorption of protein bound dye) by washing the wells of the plates five times with 1% (v/v) acetic acid. Plates were then air dried. Tris buffer (100 µL/well) was added in the plates. The plates were gently stirred for five minutes on a mechanical shaker and the optical density was recorded on ELISA plate reader at 540 nm.

The cell growth was determined by subtracting average absorbance value of the respective blank from the average absorbance value of the experimental set. The percent growth in presence of test material was calculated as under.

% cell growth = Absorbance of sample/Absorbance of negative control or untreated × 100

% growth inhibition = 100 - % cell growth.

Finally, IC_{50} values were evaluated.

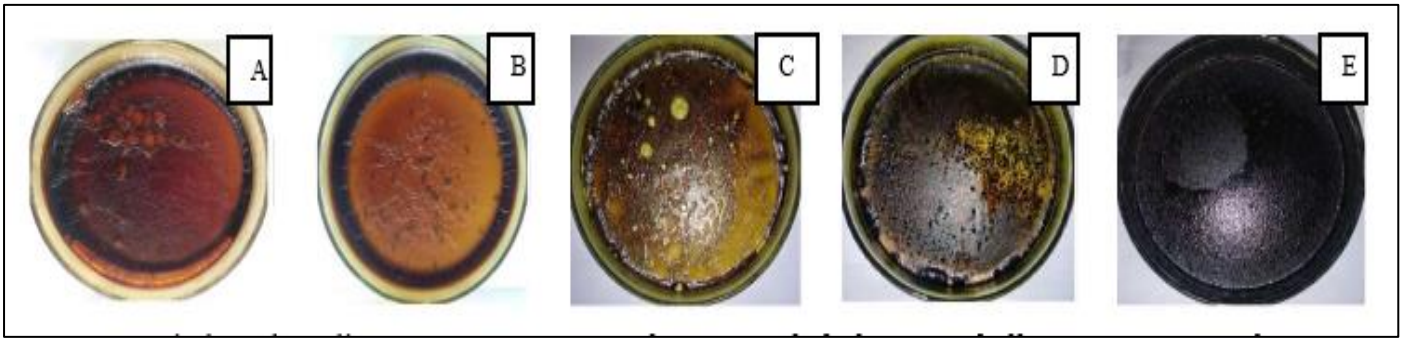

Fig 1 Dried Methanolic Extracts. A: *A. catechu*; B: *T. chebula*; C: *T. bellirica*; D: *C. tamala*; E: *O. sanctum*.

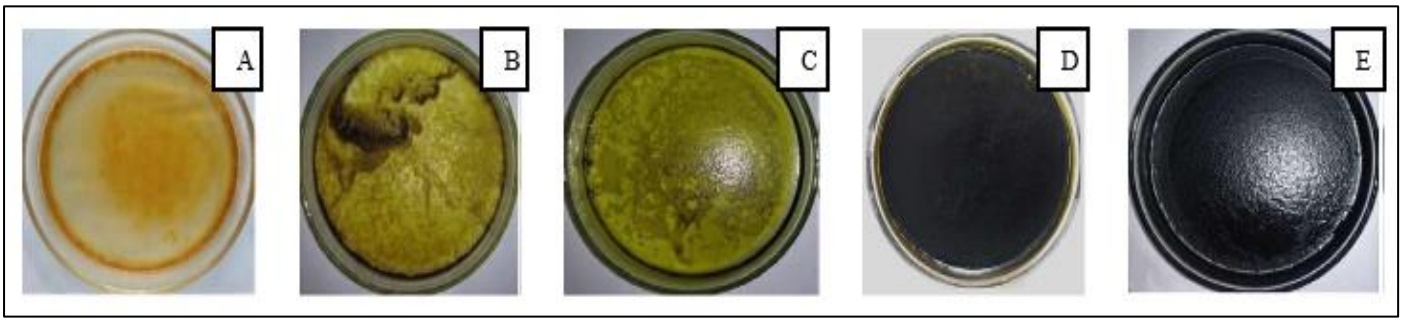

Fig 2 Dried Ethyl Acetate Extracts. A: *A. catechu*; B: *T. chebula*; C: *T. bellirica*; D: *C. tamala*; E: *O. sanctum.*

III. RESULTS AND DISCUSSION

Inflammation is an important inducer of tumor (Coussens L.M. and Werb Z. 2002). Inflammation also helps proliferation of cancer cell and involves angiogenesis and cell mobility (Mantovani A. *et al.*, 2008) Thus, reducing inflammation with the help of remedies is a therapeutic target in the treatment of cancer (Trinchieri G. 2011).

A large number of natural products posseses active metabolites that halt the key steps of the inflammation pathway such as nuclear factor kappa B, lipoxygenase and cyclooxygenase (Chouhan S. and Guleria S. 2020). The antioxidant metabolites are involved in several functional properties of fermented plant extracts viz., neutralizing free radicals, regulating antioxidant enzyme activities, reducing oxidative stress and inflammatory responses and increasing immune system performance (Shahbazi R. *et al.*, 2021). *Lactobacillus brevis* 174A mediated fermentation of *Paeoniae alba* resulted in the formation of fermented extract that involved suppression of inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) in comparison to non-fermented extract (Shakya S. *et al.*, 2021). Fermented *Laminaria japonica* extract with *Saccharomyces cerevisiae* under optimized fermentation parameters showed stronger anti-inflammatory activity in comparison to non-fermented extract (Sun Q. *et al.*, 2021).

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Consistent studies indicate that a diet rich in plant polyphenol content significantly minimizes the risk of many cancers (Dai J & Mumper R.J. 2010). Similarly, in the present study, out of seventy different non-fermented and fermented extracts of the present study, highest cytotoxic activity was shown by *A. catechu* ethyl acetate extract fermented by *L. plantarum* and *T. chebula* ethyl acetate extract fermented by *L. acidophilus* with same IC_{50} value of 71.428 µg/mL against HCT-116 cell line. Likewise, in case of PC-3 cell line, elevated cytotoxic activity was possessed by *T. chebula* non-fermented extract with IC_{50} value of 75.759 µg/mL Furthermore, *A. catechu* methanolic extract fermented by *L. acidophilus* Showed strongest cytotoxic activity against A-549 with IC_{50} value of 31.969 µg/mL. Hence, the present study suggest us that fermented plant extracts posseses higher cytotoxic activity as compared to non-fermented extracts. The cytotoxic activity of seventy different non-fermented and fermented by different probiotic microbes against three different cell lines is shown in table 1.

Table 1 Cytotoxic Activity of Non-Fermented and Fermented Plant Extracts Against HCT-116, PC-3 and A-549.

S. No.	*Extract	IC_{50} (µg/mL)		
		HCT-116	PC-3	A-549
1	ACM-NF-nutrient broth	166.666	No effect	No effect
2	ACM-NF-YPD broth	100	No effect	No effect
3	ACM-F-*Saccharomyces cerevisae*	73.529	No effect	No effect
4	ACM-F-*Bacillus subtilis*	83.333	No effect	No effect
5	ACM-F-*Lactobacillus acidophilus*	86.206	No effect	31.969
6	ACM-F-*Lactobacillus plantarum*	75.757	No effect	63.451
7	ACM-F-*Lactobacillus casei*	500	No effect	No effect
8	ACEA-NF-nutrient broth	156.25	No effect	No effect
9	ACEA-NF-YPD broth	113.636	No effect	No effect
10	ACEA-F-*Saccharomyces cerevisae*	83.333	No effect	No effect
11	ACEA-F-*Bacillus subtilis*	83.333	No effect	No effect
12	ACEA-F-*Lactobacillus acidophilus*	83.333	No effect	No effect
13	ACEA-F-*Lactobacillus plantarum*	71.428	No effect	No effect
14	ACEA-F-*Lactobacillus casei*	89.285	416.666	No effect
15	TCM-NF-nutrient broth	No effect	No effect	No effect
16	TCM-NF-YPD broth	No effect	92.592	No effect
17	TCM-F-*Saccharomyces cerevisae*	104.166	No effect	No effect
18	TCM-F-*Bacillus subtilis*	73.529	No effect	No effect
19	TCM-F-*Lactobacillus acidophilus*	80.645	No effect	No effect
20	TCM-F-*Lactobacillus plantarum*	80.645	No effect	No effect
21	TCM-F-*Lactobacillus casei*	92.592	500	No effect
22	TCEA-NF-nutrient broth	89.285	75.757	43.844
23	TCEA-NF-YPD broth	No effect	No effect	38.699
24	TCEA-F-*Saccharomyces cerevisae*	No effect	No effect	No effect
25	TCEA-F-*Bacillus subtilis*	104.166	No effect	57.603
26	TCEA-F-*Lactobacillus acidophilus*	71.428	No effect	41.528
27	TCEA-F-*Lactobacillus plantarum*	80.645	No effect	42.881
28	TCEA-F-*Lactobacillus casei*	80.645	No effect	312.50
29	TBM-NF-nutrient broth	92.592	No effect	No effect
30	TBM-NF-YPD broth	86.206	No effect	166.666
31	TBM-F-*Saccharomyces cerevisae*	No effect	No effect	No effect
32	TBM-F-*Bacillus subtilis*	No effect	500	No effect
33	TBM-F-*Lactobacillus acidophilus*	No effect	833.333	No effect
34	TBM-F-*Lactobacillus plantarum*	No effect	No effect	No effect
35	TBM-F-*Lactobacillus casei*	416.666	No effect	No effect
36	TBEA-NF-nutrient broth	833.333	2500	675.675
37	TBEA-NF-YPD broth	No effect	277.777	No effect
38	TBEA-F-*Saccharomyces cerevisae*	No effect	No effect	43.63
39	TBEA-F-*Bacillus subtilis*	2500	178.571	No effect
40	TBEA-F-*Lactobacillus acidophilus*	277.777	277.777	No effect
41	TBEA-F-*Lactobacillus plantarum*	No effect	147.058	No effect
42	TBEA-F-*Lactobacillus casei*	178.571	138.888	No effect
43	CTM-NF-nutrient broth	277.777	2500	No effect
44	CTM-NF-YPD broth	147.058	625	No effect
45	CTM-F-*Saccharomyces cerevisae*	138.888	357.142	No effect

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46	**CTM-F-*Bacillus subtilis***	1250	500	No effect
47	**CTM-F-*Lactobacillus acidophilus***	No effect	250	No effect
48	**CTM-F-*Lactobacillus plantarum***	No effect	625	No effect
49	**CTM-F-*Lactobacillus casei***	No effect	357.142	No effect
50	**CTEA-NF-nutrient broth**	No effect	500	No effect
51	**CTEA-NF-YPD broth**	No effect	250	No effect
52	**CTEA-F-*Saccharomyces cerevisae***	No effect	138.888	164.473
53	**CTEA-F-*Bacillus subtilis***	71.428	625	174.825
54	**CTEA-F-*Lactobacillus acidophilus***	No effect	138.888	No effect
55	**CTEA-F-*Lactobacillus plantarum***	No effect	108.695	No effect
56	**CTEA-F-*Lactobacillus casei***	No effect	83.333	167.785
57	**OSM-NF-nutrient broth**	No effect	2500	446.428
58	**OSM-NF-YPD broth**	No effect	2500	No effect
59	**OSM-F-*Saccharomyces cerevisae***	No effect	312.5	543.478
60	**OSM-F-*Bacillus subtilis***	No effect	108.695	1000
61	**OSM-F-*Lactobacillus acidophilus***	75.757	83.333	320.512
62	**OSM-F-*Lactobacillus plantarum***	No effect	No effect	No effect
63	**OSM-F-*Lactobacillus casei***	No effect	156.25	No effect
64	**OSEA-NF-nutrient broth**	No effect	104.166	No effect
65	**OSEA-NF-YPD broth**	No effect	178.571	139.664
66	**OSEA-F-*Saccharomyces cerevisae***	No effect	65.789	543.478
67	**OSEA-F-*Bacillus subtilis***	No effect	625	No effect
68	**OSEA-F-*Lactobacillus acidophilus***	No effect	312.5	No effect
69	**OSEA-F-*Lactobacillus plantarum***	No effect	No effect	961.538
70	**OSEA-F-*Lactobacillus casei***	No effect	No effect	No effect

- *****ACM: *A catechu* methanolic extract; ACEA: *A. catechu* ethyl acetate extract; TCM: *T. chebula* methanolic extract; TCEA: *T. chebula* ethyl acetate extract; TBM: *T. bellirica* methanolic extract; TBEA: *T. bellirica* ethyl acetate extract; CTM: *C. tamala* methanolic extract; CTEA: *C. tamala* ethyl acetate extract; OSM: *O. sanctum* methanolic extract; OSEA: *O. sanctum* ethyl acetate extract; NF: non-fermented; F: fermented.

IV. CONCLUSION

Present study concludes, that fermentation based biotransformation can be used as an alternative natural mechanisms for improvement of phytochemical constituents and bioactive properties of methanolic and ethyl acetate extracts of *A. catechu*, *T. chebula*, *T. bellirica*, *C. tamala* and *O. sanctum*. Similarly, *S. cerevisiae*, *B. subtilis*, *L. acidophilus*, *L. plantarum* and *L. casei* mediated fermentation of plant extracts under study resulted in enhancement of cytotoxic activity against HCT-116, PC-3 and A-549 cell lines which can be used in therapeutic applications.

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