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Exploring the Anti-inflammatory and Anti-cancer compounds from the leaves of *Acalypha indica*

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Abstract: *Acalypha indica (Euphorbiaceae) is commonly known as Indian Copperleaf and Indian Nettle. It is a common annual herb, which grows in the hotter parts of India like Bihar, Assam and southwards of Kerala. The hexane and aqueous extracts of leaves were screened for antioxidant and anti-inflammatory compounds as evidenced by the potent inhibition of LOX, COX enzyme activity method, which showed significant percentage of inhibition in dose dependent manner. As antioxidant therapy is found to be useful in complicated disease status related with free radical activity, the present study might be extended for the formulation and evaluation of different antioxidant herbal dosage forms. Finally Hexane extract showed potent enzymatic inhibition to COX-1 with IC50-6.48µg/ml. Ethyl acetate and ethanol soxhlation extracts IC50 values 16.43µg/ml and 40.97 µg/ml. Hexane, ethyl acetate and ethanol soxhlation extracts showed potent enzymatic inhibition to COX-2.Here aqueous extract showed moderate enzymatic inhibition. Hence the present study is taken upto systematically evaluate the anti-inflammatory and anti-cancer properties of the extracts and also to isolate and characterize the active principles of Acalypha indica.*

Keywords: *Acalypha indica, Lipoxygenase, Cyclooxygenase and Anti-inflammatory.*

I. Introduction

Medicinal plants are extensively used to cure various infectious diseases in human. Now a days, several plants have been identified for their anticancer and anti-inflammatory compounds. Scientific experiments on the anticancer properties of plants and their components have been detected. Herbal medicines have been the basis of treatment and cure for various diseases and physiological conditions in traditional methods practiciced such as ayurveda, unani and siddha. However no systematic studies were conducted to evaluate the efficacy and safety of the formulations from the plant were undertaken. Also no attempts were made to isolate and identify the active principles involved in these effects.

Inflammation

Inflammation (Latin, *inflammare*, to set on fire) is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero-Miliani *et al.,* 2007*).* Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Without inflammation, wounds and infections would never heal. Similarly, progressive destruction of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases, such as hay fever, atherosclerosis, and rheumatoid arthritis. It is for that reason that inflammation is normally closely regulated by the body. Inflammation can be classified as either *acute* or *chronic*. Acute inflammation is the Initial response of the body to harmful stimuli and is achieved by the increased movement of leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Causes

- Burns, Chemical irritants
- Frostbite
- Toxins
- Infection by pathogens
- Physical injury, blunt or penetrating
- Immune reactions due to hypersensitivity
- Ionizing radiation

• Foreign bodies, including splinters, dirt and debris

Eicosanoids

In mammalian cells, Eicosanoid biosynthesis is usually initiated by the activation of phospholipase A_2 and the release of Arachidonic acid (AA) from membrane phospholipids. The Arachidonic acid is subsequently transformed by cycloxygenase (COX) and Lipoxygenase(LOX) pathways to prostaglandins, thromboxane and leukotrienes. Eicosanoid production is considerably increased during inflammation. Eicosanoids, the oxygenated metabolites of eicosapolynoic fatty acids such as AA, have been the most actively studied of all physiological components contributing to inflammation (Williams and Higgs. 1988, Wymann and schneiter, 2008).AA generated from cellular membrane phospholipids gets oxygenated by either the COX pathway that generates prostaglandins (PGs) or the LOX pathway that forms hydroperoxy derivatives. In addition to COX and LOX pathways, AA is also oxygenated by the epoxygenase pathway involving cytochrome P^{45S} to generate Epoxyeicosatrienoic acids (EETs).

II. Materials & Methods

Plant collection: Mature leaves of *Acalypha indica* were collected from Tirumala at Chittoor district, Andhra Pradesh. Leaves were washed thoroughly three times with water and once with distilled water. The leaves were shade dried and powdered by grinder machine. The powdered samples were sealed in polythene bags until the time of extraction.

***In Vitro* Enzyme assays**

For Screening of extracts against to 5-LOX, 15-LOX and COX-1, COX-2 , the concentrations were taken in the respective way,100 µg/mL, 10 µg/mL and 1 µg/mL as final concentration.

***In vitro* 5-LOX and 15-LOX Enzyme assays**

Polarographic method was used to measure the enzyme activity with a CLARK's method oxygen electrode on Gilson model 5/6 oxygraph. Typical reaction mixture contained 3ml with assay buffer, distilled water and enzyme and AA. Since Lipoxygenases are oxygen-consuming enzymes, the concentration of oxygen decrease in the reaction mixture and the rate of decrease in oxygen were taken as a measure of enzyme activity. Reaction was allowed to proceed at 25°C and from the curve recorded the difference in oxygen concentration per minute was taken for calculating enzyme activity. The initial and final concentrations were recorded on the oxygraph. One unit of enzyme activity is defined as 1µmole of oxygen consumed/min and IC_{50} values were calculated. Typical reaction mixture contains.

1. 150mM phosphate buffer (pH 6.3)- 2 mL
2. Distilled water -910 µl
3. Enzyme (5-LOX/15-LOX) -50 µl
4. Compound (crude extract) -30 µl
5. Arachidonic acid -40 mM 10 µl

Total- 3 ml

Enzyme activity was calculated using the equation:

ENZYME ACTIVITY= [(Micromole O_2 incorporated/min)/ml of enzyme used**]**

µMole of O2 incorporated (µ moles/min/ml) = [Initial O_2 conc. – Final O_2 conc**.**]

Reaction mixture for enzyme activity

Buffer 2 mL + Water-940 µl + Enzyme-50 µl + AA 10 µl

Reaction mixture with extract

Buffer 2 ml + Water-910 µl + Enzyme-50 µl +Extract-30 µl + AA 10 µl

Cell culture and treatment

Cell Culture

The human squmous skin cancerous cell line A431 was obtained from National Centre for Cell Science (NCCS), Pune, India and maintained in DMEM supplemented with 10% heat inactivated FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-Glutamine and maintained in a humidified atmosphere with 5% CO_2 at $37^{\circ C}$. The cells were sub-cultured twice each week and the exponentially growing cells were used in all treatments. Different extracts of *A. indica* dissolved in DMEM and DMSO respectively were used in the treatments. 10mg/mL stock of extracts was employed in this study.

Treatment of cells

At the time of treatment, working solutions were diluted accordingly in DMEM. Final concentrations of different extracts were ranged from 1µg/mL to 500 µg/mL and . The drugs were added to the cells, 24hr of seeding in 100mM Petri dish. The final concentration of the vehicle (DMSO) never exceeded 0.05%. A431 cells exposed to 0.1% DMSO served as controls.

III. Results & Discussion

Yields of *Acalypha indica leaf* extracts

100gm of shade dried leaf powder of *Acalypha indica* had yielded the following amount of each of the extracts when the solvents, hexane, ethyl acetate, methanol were used in the increasing order of polarity from hexane to water during Soxhlation and soaking method.

Table 1: Yields of *Acalypha indica leaf* extracts

Plant	Solvent	Extract after evap./lyoph.(gm)
Acalypha indica	Hexane (Soxhlation)	3.46
	Ethyl acetate (Soxhlation)	0.14
	Ethanol (Soxhlation)	11.54
	Ethanol (soaking)	5.86
	Methanol (Soxhlation)	4.85
	Methanol (soaking)	9.66
	Aqueous(soaking)	13.1

Table 2: Inhibition 5-LOX by various extracts of *Acalypha indica* leaves.

	Conc. (µ g/ml)	Initial O_2 conc.	Final (µ mol/min)	Difference.	Avg.	Enzyme activity (µmol O_2incorporated/min/ ml)	
Control	_	a.239.84 b.250.05	a.56.32 b.61.66	a.183.52 b.188.39	185.95	25.66	
Sample	Conc. (µ g/ml)	Initial o_2 conc.	Final (µ mol/min)	Difference.	Avg.	% Activity	% Inhibition
NDGA	10	249.63	193.32	56.31	_	30.24	69.76
NDGA	1	253.58	110.62	172.71	_	92.88	7.12
HX	100	a.195.90 b.198.24	a.67.43 b.83.89	a.128.47 b,114.35	121.49	65.29	34.71
EA	100	a.228.64 b.231.83	a.112.53 b.115.78	a.116.11 b.116.05	116.08	62.68	37.32
AQ	100	a.256.24 b.259.02	a.76.37 b.85.44	a.179.87 b.173.58	176.73	95.43	4.57
Et.SK	100	a.203.23 b.197.30	a.68.15 b.75.69	a.135.08 b.173.58	128.34	69.30	30.70

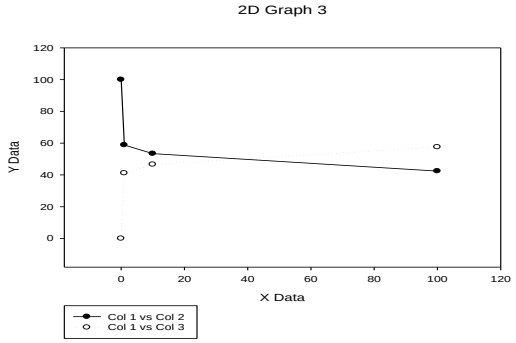
Table 3: 5-LOX inhibition by various extracts of *Acalypha indica* leaves.

Sample	Conc. (µ g/ml)	Initial o_2 conc.	Final (µ mol/min)	Difference.	Avg.	Enzyme activity (µmol O_2incorporated/min/ ml)	
Control	_	a.202.2 b.203.92	a.61.43 b.85.92	a.141.97 b.118	129.99	17.94	
Sample	Conc. (µ g/ml)	Initial o_2 conc.	Final (µ mol/min)	Difference.	Avg.	% Activity	% Inhibition
NDGA	10	200.79	153.01	47.78	_	36.19	63.81

NDGA	1	203.92	85.92	118	_	90.86	9.14
ETSX	100	a.183.78 b.179.89	a.70.86 b.56.02	a.112.92 b.123.87	118.34	91.12	8.88
MTSK	100	a.149.11 b.162.73	a.45.53 b.62.56	a.103.58 b.100.11	101.85	78.42	21.58
MTSX	100	a.194.52 b.194.86	a.83.95 b.106.75	a.110.57 b.88.1	99.33	76.48	23..52

Table 4: 15-LOX inhibition by various extracts of *Acalypha indica* leaves.

Concentration µg/ml	%Activity	%Inhibition	IC_{50} Values
Ethanol (Soxhlation) Extract of *Acalypha indica*			
1	58.84	41.16	39.2079
10	53.4	46.60	
100	42.5	57.55	
Ethanol (Soaking) Extract of *Acalypha indica*			
1	32.07	67.93	28.3045
10	31.77	68.23	
100	29.88	70.8	



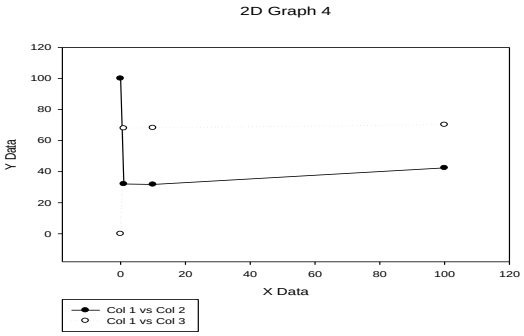


Fig.1: Effect of ethanol (sox) extract on 15 LOX activity **Fig.2: Effect of ethanol (soaking) extract on 15 LOX activity**

X axis-Concentration of extracts in µg/ml
Y axis-%Enzyme Activity/inhibition

Table 5: Enzyme Activity of COX 1 and COX 2

Enzyme Name	**Activity(Mol/min/mL)**
COX 1	95.157
COX 2	34.50

Table 6: COX-1 Inhibition by various extracts of *Acalypha indica*

Concentration(g/ml)	**%Activity**	**%inhibition**	**IC_{50} Values**
Ethyl acetate extract of *Acalypha indica*			
1	67.43	32.57	16.43
10	51.90	48.10	
100	27.74	72.26	
Hexane Extract of *Acalypha Indica*			
1	69.72	30.28	6.48
10	37.66	62.34	
100	13.23	86.77	
Ethanol(Soxhlation)extract of *Acalypha indica*			
1	84.73	15.27	40.97
10	61.23	38.68	
100	28.24	71.75	

Table 7:COX-2 Inhibition by various extracts of *Acalypha indica*

Sample	Concentration(µg/mL)	%Activity	%Inhibition
Ethylacetate ext.	100	30.53	69.47
Hexane ext.	100	30.53	69.47
Ethanol Sx.ext.	100	68.77	31.23

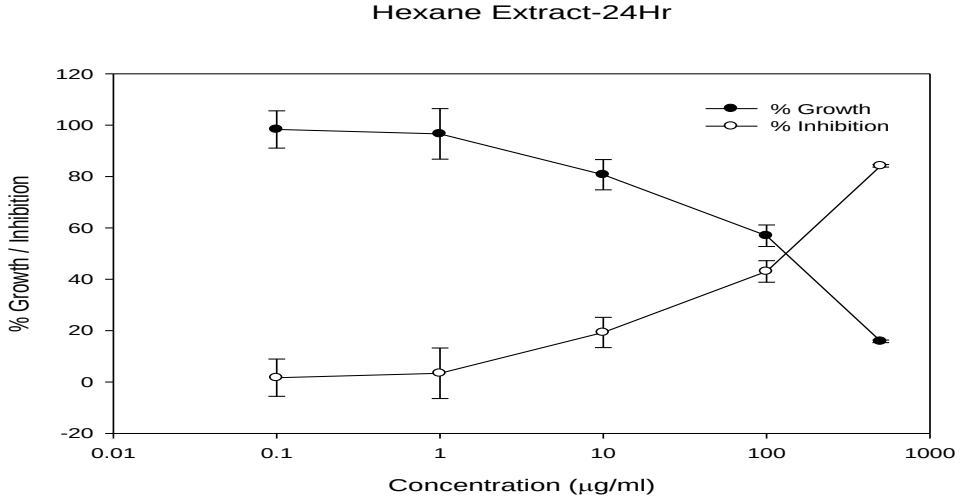


Fig 3: Measurement of effect of hexane extract of *Acalyppha indica* on the growth of A431 cells by MTT assay

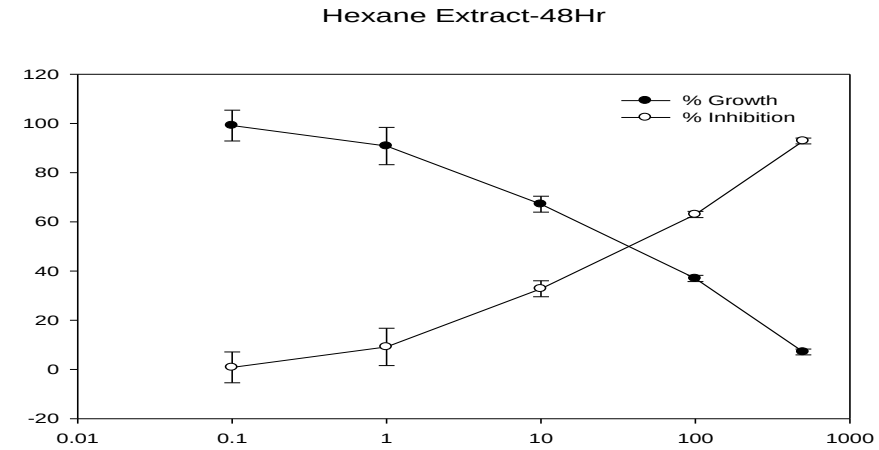


Fig.4: Measurement of effect of hexane extract of *Acalyppha indica* on the growth of A431 cells by MTT assay

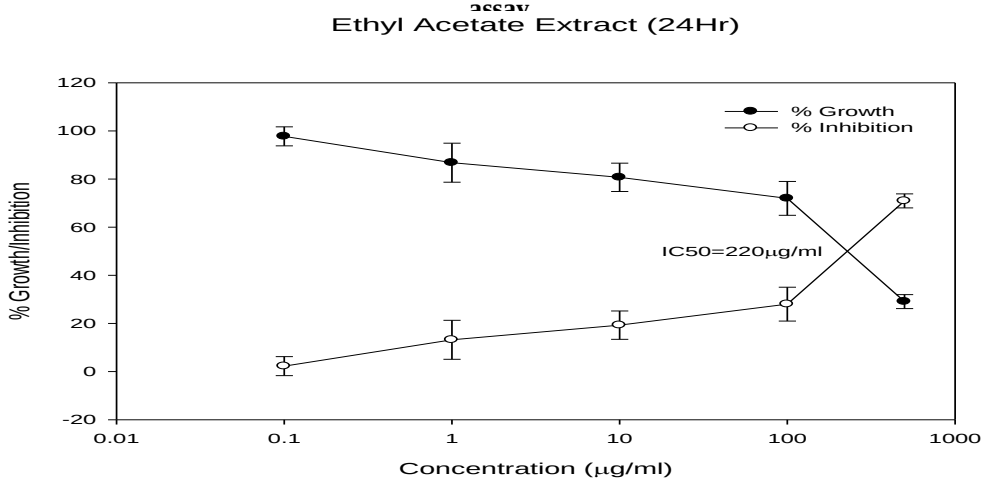


Fig.5: Measurement of effect of ethyl acetate extract of *Acalyppha indica* on the growth of A431 cells by MTT assay

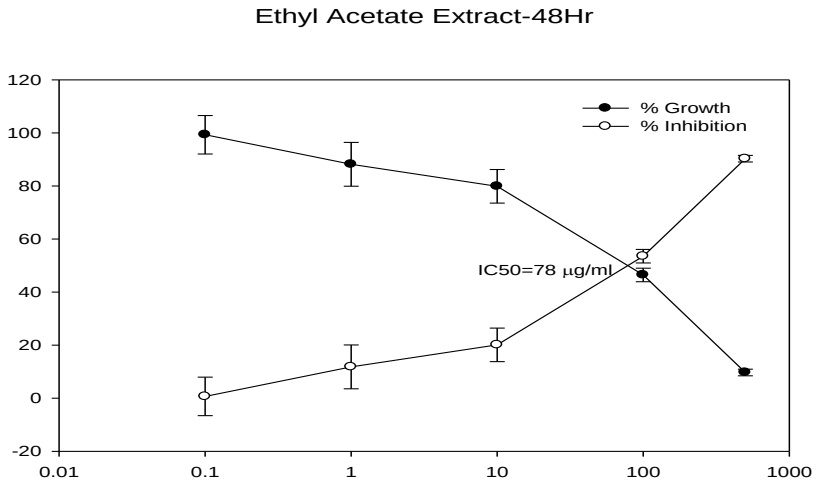


Fig.6:Measurement of effect of ethyl acetate extract of *Acalyppha indica* on the growth of A431 cells by MTT assay

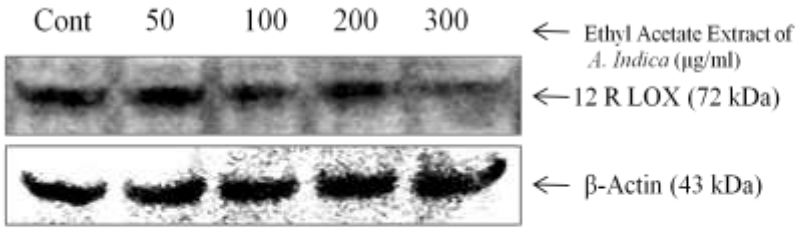


Fig: Western blot showing the effects of Ethyl Acetate extract of *A. Indica* on expression of 12 R LOX in whole cells lysates of A431 cell line. Cells were incubated with different concentrations of compound (50 100, 200 and 300µg/ml) for 24Hr. β -Actin was used as an internal control.

IV. Discussion

The use of traditional medicinal plants in most developing countries for the maintenance of good health has been widely observed. *A.indica* is a traditional medicinal plant in India, used as antibacterial, anti-fungal activities. It is observed that this plant has anti-inflammatory and anti-cancer properties. It is also used to treat diabetes and other diseases and disorders.

Eicosanoids are potent biologically active AA derived lipid mediators that are intimately involved in inflammation and cancer. COX and 5-LOX are the key enzymes in the AA metabolism. COX enzyme exists in two distinct isoforms, COX-1 and COX-2. COX-1 is expressed constitutively in nearly all mammalian tissues and is the source of prostaglandins central to "housekeeping" functions such as renal water reabsorption, vascular homeostasis, and gastric protection (Smith *et al.,* 1996). COX-2, an inducible enzyme, is mainly expressed under pathological conditions such as inflammation and carcinogenesis.

In the light of key role played by COX-2 in inflammation, COX-2 Inhibitors were developed as novel NSAIDs without gastric side effects that are associated with the conventional NSAIDs. In addition to their role in inflammation, selective inhibitors of COX-2 have been demonstrated to induce apoptosis in variety of cancer cell lines including colon, stomach, prostate, and breast (Elder *et al.,* 1997). 5-LOX is involved in the biosynthesis of LTs, pro-inflammatory mediators participating in various forms of acute and sub acute inflammation. 5-HETE, product of 5-LOX was shown to be a potential survival factor for prostate cancer cells and inhibition of 5-LOX triggered massive apoptosis (Ghosh *et al.,* 1998). Both 5-LOX and COX-2 are co-expressed and up-regulated in inflammation and in many forms of human cancers, including colon, prostate, breast and lung cancers and form targets for development of anti-inflammatory and anti-cancer drugs (Pommery *et al.*, 2004). Moreover, COX-2 inhibition by celecoxib in cancer cell lines was shown to increase the formation of 5-HETEs, which is having tumor cell proliferative property (Ye *et al.,* 2005). Hence there is need for the development of potent COX-LOX dual inhibitors without side effects.

In our attempt to isolate a natural product with COX-LOX dual inhibition, *Acalypha indica* is identified as a potential source. The studies with different extracts of *Acalypha indica* showed potent inhibition to 5-LOX, 15-LOX and COX-1. The 5-LOXinhibition of four extracts of *A.indica* in increasing order is as follows:

aqueous extract <ethanol (sx) extract < methanol (sk) extract < methanol (sx) extract < ethanol (sk) extract < hexane extract < ethyl acetate extract. In the present study, our results have shown that extracts of inhibited 15-LOX. Ethanol soxhlation and soaking extract showed more potent inhibition when compared to ethyl acetate and hexane extracts. The 15-LOX inhibition of four extracts of *A. indica* in increasing order is as follows: water extract < hexane < ethyl acetate extract < ethanol extract. Hexane, ethyl acetate and ethanol soxhlation extracts showed potent inhibition to COX-1. Among this hexane extract showed potent enzymatic inhibition to COX-1 with IC50 – 6.48µg/ml. Ethyl acetate and ethanol soxhlation extracts' IC50 values 16.43µg/ml, 40.97µg/ml respectively. Here aqueous extract showed moderate enzymatic inhibition. The COX-1 inhibition of four extracts of *A. indica* in increasing order is as follows: water extract < ethanol (sx) extract < ethyl acetate extract < hexane extract. Hexane, ethyl acetate and ethanol soxhlation extracts showed potent inhibition to COX-2. Among this hexane extract and ethyl acetate showed potent enzymatic inhibition to COX-2. COX-2 inhibition of 3 extracts of *A. indica* in increasing order is as follows- ethanol (sx) extract < ethyl acetate and hexane extract.

From the above results it is observed that the ethyl acetate extract and hexane extract of *A. indica* showed potent enzyme inhibition to 5-LOX and COX-2 enzymes when compared to 15-LOX enzyme. Hexane extract of *A. indica* showed potent enzyme inhibition of COX-1 enzymes. Ethanol (sx) extract of *A. indica* showed potent enzyme inhibition to both 15-LOX and COX-1, no inhibition to 5-LOX enzyme.

V. Conclusion

The present study reveals that the leaves of *A. indica* contain potent anti-inflammatory compounds as evidenced by the potent inhibition of lipoxygenases and cyclooxygenases. These compounds appear to be non-polar in nature as they were extracted in to non-polar solvents like hexane, ethyl acetate and ethanol, methanol. Further studies, however, are needed to identify the specific molecules involved in the inhibition of these enzymes.

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