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GC-MS Analysis, Antipyretic, and COX-2 Inhibitory Activity of Traditional Fermented Formulation Ushirasav

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ABSTRACT

Ushirasav is a fermented traditional polyherbal formulation of modern Ayurveda, it is used to treat heat generate disorders in the body like bleeding of the nose and rectum, in this research Ushirasav is prepared according to the method given in Ayurvedic pharmacopeia of India by a controlled fermentation process and it was strictly observed for the presence of ethanol in a prescribed amount and the absence of methanol. Then Ushirasav was exposed to GC-MS analysis for exploration of types of metabolites present in the formulation and their reported effects, based on traditional claims, Ushirasav was subjected to *in vivo* antipyretic potential. It was found that Ushirasav showed antipyretic potential as compared to standard drug Paracetamol. Then by GCMS, analysis the prime content of Ushirasav is found as Benzoic acid. Benzoic acid was docked on Cyclooxygenase enzyme II for exploration of antipyretic potential. Benzoic acid bind with COX II and its binding energy were comparable to Indomethacin. Thus, it was explored from this research that Ushirasav shows remarkable antipyretic activity and it may be attributed to presence of Benzoic acid.

Keywords: COX-2, Fermentation, GCMS, Pyrexia, Traditional, Ushirasav.

INTRODUCTION

Ushirasav is a fermented Ayurvedic formulation that is included in Ayurvedic Pharmacopoeia of India. It is used in urinary tract infections, anemia, swelling, and disorders generated in the body due to excessive heat.[1] It contains Ushir (Vetiveria zizanioides) in addition to water, sugar, and honey. Twenty-four herbs are also present for synergistic effects due to the complex nature multitargeted approach. All herbs are present in equal amounts.[2] Pyrexia is a symptom induced in the body due to excessive heat. Paracetamol is a well-known antipyretic but its hepatotoxicity is also a major factor for the search of herbal alternatives. Ushirasav is prepared and standardized according to the Ayurvedic Pharmacopeia of India.[3] Indomethacin, a well-known NSAID, has been widely used for its anti-inflammatory and analgesic properties through its action on COX-2. However, its use is often limited by adverse effects and the need for improved selectivity. Over the past few years, there has been a heightened interest in designing new molecules that can provide enhanced COX-2 selectivity and better therapeutic profiles.

This manuscript explores the molecular interactions between COX-2 and indomethacin, along with molecule found in major amount in Usirasav: Benzoic acid. Utilizing docking simulations, we aim to elucidate the binding affinities and interactions of this compound with COX-2. By comparing the binding modes of these novel compounds with those of indomethacin, we seek to identify potential improvements in selectivity and efficacy. This study not only provides insights into the binding mechanisms of these molecules but also contributes to the rational design of future COX-2 inhibitors with optimized therapeutic profiles.

MATERIAL AND METHOD

Formulation of Ushirasav: Ushirasav was prepared according to the methodology given in Ayurvedic Pharmacopoeia of India.[4]

Plant Material: All plant materials were purchased from the local Nursery at Gagan, Moradabad, and authenticated by botanist Dr. Ashok Kumar, School of Sciences, IFTM University, Moradabad, and voucher specimens of plants were kept there for future references.

Chemical and reagents

Indomethacin (CDH, New Delhi, Brewer's yeast (Sigma-Aldrich, USA), Paracetamol (GSK, Nashik).

Methodology for Ushirasava formulation

For preparing Ushirasav *Vetiveria zizanioides(rt), Coleus vettiveroides(rt), Nelumbo nucifera (fl), Gmelina arborea(Bk.), Nymphaea stellate(fl.), Callicarpa macrophylla (fl), Prunus cerasoides(st.), Symplocos racemose(bk.), Rubia cordifolia(rt), Fagonia cretica(pl), Cissampelos pareira(rt), Swertia chirata(pl), Ficus benghalensis (st.bk.), Ficus racemose(st.bk.), Hedychium spicatum(rh), Fumaria parviflora(pl), Nelumbo nucifera (fl), Trichosanthes dioica(lf), Bauhinia variegate(st.bk.), Syzygium cumini(st.br), Salmalia malabarica(exu.)*, were taken in equal amounts (48 g). Then a coarse powder of above materials was created and these were cleaned, dried, and then put through sieve number 44 and then mixed. *Vitis vinifera*(fr) (960g) was cleaned and sanitized, and 4.8 g sugar was dissolved in a specific amount of water and then filtered through muslin cloth. The filtrate was placed in a sanitized mud container and mixed in the final medications, Honey (2.4 g), Vitis *Vinifera*(fr), and 768 g *Woodfordia fruticose* (fl.), Water(24.56L), Sugar, and Piper nigrum(q.s.) were added and then the container's mouth was closed. The container was moved into the fermentation area and kept there and observed for indications that the fermentation process was nearing its end. The fermented substance was passed through a muslin cloth that had been cleaned. It was placed in airtight receptacles to mature. The initiation of fermentation was confirmed by presence of bubbles and a crackling sound, which vanish on completion. It took one and the half months for Ushirasav to prepare.

Evaluation for the absence of methyl alcohol: It was evaluated by oil of wintergreen test.

Oil of Wintergreen Test

5 mL of sample Ushirasava, poured in a test tube, and a pinch of salicylic acid and 3 drops of sulphuric acid were added to it. The test tube was kept in boiling water for a few minutes and the Ushirasav was poured into another test tube, which contained five ml of dilute sodium carbonate solution. No formation of a pleasant smell of methyl salicylate was noted which indicates the absence of methanol in the Ushirasav.[5]

In the traditional fermented formulation quantity of Ethanol is analyzed by distillation method.

Quantification of Ethanol

25 milliliters of Ushirasav was placed in a distillation flask to estimate the alcohol concentration. After adding a small amount of pumice powder in the distillation flask and then the sample was diluted up to 150 milliliters of water, the distillation head and condenser were connected. When the volumetric flask got 90 mL of distillate, it was cooled to 25°C.A 100 mL volume adjustment was made. Following that, the sample's specific gravity was measured, and the alcohol content was calculated using the chart provided in Indian Pharmacopoeia.[6]

Phytochemical revelation

The secondary metabolites such as

phenolics, alkaloids, glycosides, flavonoids were revealed in Ushirasav by various qualitative tests.[7]

Statistical analysis: All calculations were executed by GraphPad Prism software and data is presented as ±SD. All data has a significant p-value.

GCMS Analysis: Ushirasav is subjected to analysis of volatile components with GCMS approach using gas chromatographic approach equipped with a mass spectrometer. As on electronic bombardment chemical components lose valency electrons from the outer shell and are ionized as m/z where m is the mass of the atom while z is its charge. Ushirasav sample was prepared by fractionationed 5 mL it in 100 mL of nHexane and Ethyl acetate(1:1) using separation funnel. Solvent phase was collected for study, then moisture was removed by sodthiosulphate and the sample was concentrated by rotatory evaporater. For GCMS analysis Agilent 7890 instrument equipped with flame ionization detector was used.The capillary column was 30 meter long and 0.25mm thick internally,coated with film of stationary phase. Carrier gas was Helium at a flow rate of 1mL/min and temperature at the time of injection of sample was 60°C and it rise at the rate 10°C up to 300°C Data is processed by Mass Hunter software. The spectra are compared against databases by the NIST library for compound identification. Fifty-four peaks were obtained by individual compounds. Quantification was done by peak area while indicator of abundance is by peak height.

Antipyretic potential: The antipyretic potential of Ushirasav is evaluated in yeast-induced pyrexia model.

Animals

Wistar albino rats weighing 150–200 g were kept for the preclinical trials at the IFTM University animal house in Moradabad, Uttar Pradesh, India. Each animal was safely kept in clean polypropylene cages with alternating light and dark cycles every 12 h at a consistent temperature of 24±1°C. The animals were given unfettered access to water and a balanced diet of regular pellets (Hindustan Lever Ltd., India). The Institutional Animal Ethical Committee (IAEC, reference number IAEC/2021/33) approved the animal experimentation Protocol.

Acute toxicity and Antipyretic test

Ushirasav was administered orally at the dose level up to 10 mL to six albino wistar rats according to OECD 423 guidelines. They were observed for sign of toxicity up to 24 hours. No toxicity was observed so 2 mL and 4 mL were chosen as low and high dose.

For antipyretic efficacy, Wistar albino rats weighing 150–200 g was accustomed. Before the experiment began, the chosen animals were healthy and acclimated to the lab environment. Four sets of six rats each were created from the animals. After taking a digital thermometer reading of each rat's body temperature, a 20% aqueous suspension of Brewer's yeast (10 mL/kg, s.c.) was injected into each rat to induce pyrexia. Every rat's rectal temperature was taken a day after each group's overnight fast, during which time they were all given unrestricted access to drinking water. A temperature increase of more than 0.5°C was required to confirm the induction of pyrexia, and animals exhibiting a temperature rise of less than 0.5°C were not allowed to continue in the experiment.[8] All four groups animals are administered with normal saline, Paracetamol, Ushirasav 2 mL/Kg and 4 mL/Kg body weight. The rectal temperature was measured at the time of administration of dose and after 1,2,3 and 4 hour.

COX-2 Inhibitory potential

It outlines the systematic approach for conducting docking studies using AutoDock Vina to explore the interactions of COX-2 with Indomethacin (as a positive control) and, Benzoic acid (as the query compounds), aiming to uncover their binding affinities and potential therapeutic implications.

Preparation of Protein and Ligands: 3D structure of Protein COX-2 was considered as PDB 5kir. PDB was downloaded as the COX-2 structure (PDB ID: 5kir) from the Protein Data Bank (PDB). Protein was prepared by eliminating water molecules, heteroatoms, and any co-crystallized ligands. Hydrogen was used for valency management. 3D structure of ligands i.e. Indomethacin & sample molecules were prepared for the ligand by adding

charges and optimizing its geometry using molecular modeling software.

Docking Setup: We used AutoDock Vina for performing molecular docking simulations. Grid box was defined around the active site of COX-2 to encompass potential binding sites. The exhaustiveness parameter was set-up to an appropriate value to ensure a thorough exploration of binding conformations. Docking Protocol receives Input Files: as the configuration file for each ligand, the protein (COX-2), and coordinates of the grid box, exhaustiveness with other parameters in the docking configuration file.

AutoDock Vina was executed for each ligand-protein docking interaction separately. Docking scores was recorded (binding affinity) and predicted binding modes (poses) of each ligand. Post-Docking Analysis was performed to identify the most favorable binding poses and corresponding binding energies (interactions between COX-2 and each ligand was visualized using molecular visualization tools to save images. The docking scores were compared and interactions between Indomethacin (positive control) and sample query compounds to assess their binding affinities and potential modes of interaction with COX-2. Query interaction was observed in relation to positive control.

As benzoic acid is confirmed in Ushirasav in major amounts by GCMS analysis. So it was a query molecule, docked against receptor COX-2 for antipyretic efficiency and the standard drug was COX-2 inhibitor Indomethacin.[9] These studies were performed by molecular docking software Autodock Vina.

RESULTS AND DISCUSSION

The results obtained from the above studies are summarized below

Methanol content: Methanol is a toxic compound and in alcoholic fermented formulations it is necessary to confirm its absence. As smell of methyl salicylate was not produced in the test. It was confirmed that methanol is not present in the formulation.

Ethanol Content

It was found 5.64% in Ushirasav. So, it is in limit and alcohol is necessary in this formulation.

Phytochemical analysis:

Phytochemical analysis of Ushirasav shows the presence of alkaloids, glycosides, sugars, flavonoids, and phenolics.

GC-MS analysis: The GCMS total ion chromatogram of Ushirasav is shown in

NIST search report of Ushirasav is shown in Table 1.

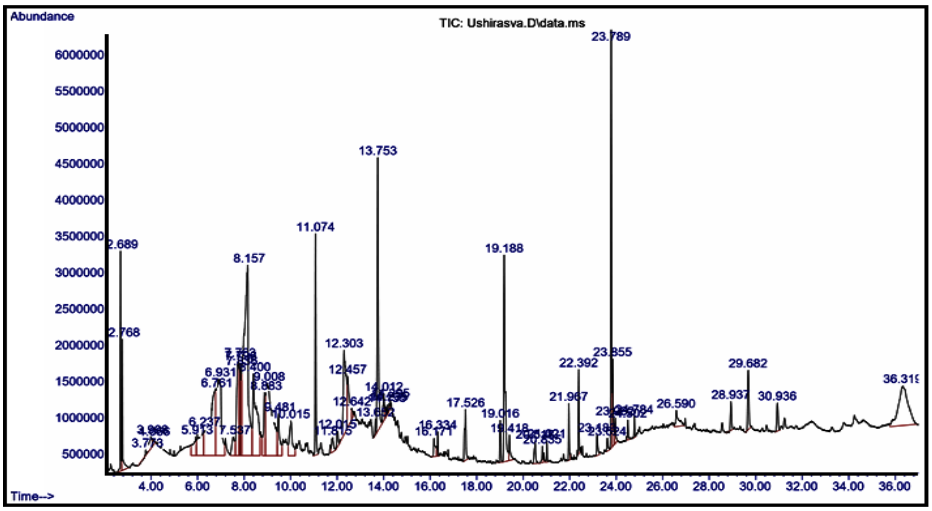

Fig. 1. GCMS graph of Ushirasava (Image is obtained from GCMS analysis of Ushirasav from Jamia Hamdard, New Delhi Central Instrumentation Facility., Some of the peaks are very close, so they appear to be overlapped)

Table 1: The GC MS analysis of Ushirasav shows the major presence of the following compounds as documented by NIST library

Sr. No	Peak	Area	CAS No.	Compound	Therapeutic use
1	2.689	1.60	024347-58-8	2,3 Butanedieol	-
2	2.768	1.27	000513-85-9	2,3-Butane-2,3-diol,	-
3	3.773	0.02	043044-24-2	Thietane, 2,4-dimethyl- Butanoic acid	Anti-inflammatory[10]
4	3.998	0.53	997376-73-5	2-Imino-6-nitro-2H-1-benzopyran-3- carbothioamide	Antiarrthymic activity[11]
5	4.066	0.17	043044-24-2	Thietane, 2,4-dimethyl- Thiophene,	-
6	5.913	0.94	119968-53-5	2-Diisopropylsilyloxypropane	-
7	6.237	1.88	000075-35-4	Ethene	-
8	6.761	6.60	002319-57-5	1,2,3,4-Butanetetrol	-
9	6.931	6.44	002319-57-5	1,2,3,4-Butanetetrol	-
10	7.537	0.88	005336-24-3	Urea	Promote digestion, and improve hepatic function. induced apoptosis of tumor cells ,neuroprotective properties[12]
11	7.763	3.39	028564-83-2	4H-Pyran-4-one, 2,3-dihydro-3,5-di hydroxy-6-methyl-	Antioxidant
12	7.796	1.01	028564-83-2	4H-Pyran-4-one, 2,3-dihydro-3,5-di hydroxy-6-methyl-	
13	7.836	1.88	028564-83-2	4H-Pyran-4-one, 2,3-dihydro-3,5-di hydroxy-6-methyl	
14	8.157	14.41	000065-85-0	Benzoic acid	Antimicrobial[11]
15	8.400	5.06	000098-55-5	alpha.-Terpineol	Antioxidant[13]
16	8.883	1.64	000067-47-0	5-Hydroxymethylfurfuralr	-
17	9.008	7.87	000067-47-0	5-Hydroxymethylfurfural	Antioxidant
18	9.481	1.43	000099-94-5	Benzoic acid, 4-methyl-	-
19	10.015	1.72	997032-27-9	3-Hydroxythiophenol	-
20	11.074	2.64	000088-04-0	Chloroxylenol	Antimicrobial[14]
21	11.815	0.46	997920-01-5	1-[(5-Nitroindl-2-yl)carbonyl	-
22	12.015	0.36	033933-82-3	2-Decanone, 5,9-dimethyl	-
23	12.303	3.76	063785-74-0	Cyclopenta[c]pyran-4-carboxylic ac id, 7-methyl-, methyl ester	-
24	12.457	1.83	063785-74-0	Cyclopenta[c]pyran-4-carboxylic ac id, 7-methyl-, methyl ester	Anticancer
25	12.642	0.20	063785-74-0	Cyclopenta[c]pyran-4-carboxylic	-
26	13.652	0.18	997175-26-9	3,7-Dimethyl-2,3-epoxy-1,2,3,4-tet rahydro-.1.beta.-naphthol	-
27	13.753	4.93	063785-74-0	Cyclopenta[c]pyran-4-carboxylic acid, 7-methyl-, methyl ester	-
28	14.012	0.57	002046-76-6	3-Acetyl-5-methylbenzo(b)thiophene	-
29	14.155	0.27	077130-65-5	1,3,6,7-Tetrahydro-1,1-dimethylcyclo buta[q]2,1-benzoxasilole	-
30	14.235	0.04	016587-45-4	Benzo[b]thiophene	-
31	14.295	0.10	997098-02-3	3-Deoxy-d-mannoic lactone	-
32	16.171	0.47	000504-96-1	Neophytadiene	Anti-inflammatory[15]
33	16.334	0.24	000504-96-1	Neophytadiene	-
34	17.526	1.11	000057-10-3	n-Hexadecanoic acid	-
35	19.016	0.60	000150-86-7	2-Hexadecen-1-ol, 3,7,11,15-tetram ethyl	-
36	19.188	4.20	000060-33-3	9,12-Octadecadienoic acid	-
37	19.418	0.46	000057-11-4	Octadecanoic acid	Anti-viral antibacterial, antioxidant
38	20.518	0.52	040817-19-4	Dimethyl aminoethyl palmitate	Anti-inflammatory[16]
39	20.835	0.22	000947-05-7	Oxacyclotridecan-2-one	-
40	21.021	0.24	035281-25-5	9,14-Dihydrobenzo[b]triphenylene	-
41	21.967	0.62	997419-89-8	2-(Dimethylamino)ethyl (9Z,12Z)-octadeca-9,12-dienoate	-
42	22.392	0.79	023470-00-0	Hexadecanoic acid	-
43	23.183	0.39	000481-74-3	9,10-Anthracenedione, 1,8-dihydrox	
44	23.624	0.23	997503-88-2	Negletein	-
45	23.789	4.47	003443-82-1	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyle Ster	-
46	23.855	1.31	003443-82-1	9,12-Octadecadienoic acid	-
47	23.962	0.37	000621-61-4	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	-
48	24.502	0.27	055751-83-2	2-Ethylacridine	-
49	24.784	0.21	288246-53-7	Pyridine-3-carboxamide	
50	26.590	0.75	997453-21-7	1-(3-Chlorophenyl)-5-phenyl-1,2,3-triazol-4-amine	
51	28.937	0.49	997453-21-7	1-(3-Chlorophenyl)-5-phenyl-1,2,3- triazol-4-amine	Anti-inflammatory, anticancer
52	29.682	1.16	000083-47-6	Gamma.-Sitosterol	Anti-cancer[17]
53	30.936	0.62	997453-21-7	1-(3-Chlorophenyl)-5-phenyl-1,2,3- triazol-4-amine	
54	36.319	6.27	997394-71-6	acetic acid,	Antiseptic[18]

#Especially, the hyphenation with MS provides reliable information for qualitatively analyzing complex constituents., the major constituent by GCMS analysis is Benzoic acid as it occupies area of 14.41percent

Acute toxicity studies: According to OECD 423 guidelines, Ushirasav when administered to unisex Wistar albino rats in doses up to 10 mL/Kg body weight doesn't show any toxic effect.[19]

Anti-pyretic potential: The antipyretic potential of Ushirasav was evaluated by Yeast induced pyrexia method as described in Table 2.

Table 2: Antipyretic Study of Ushirasav

Treatment	Dose (mL/kg)	Time				
		0 h	1 h	2 h	3 h	4 h
		Rectal temperature (°F)				
Control		98.58±0.19	101.33±0.27	101.45±0.48	101.58±0.24	101.50±0.22
Paracetamol	100 mg/Kg	98.46±0.32**	101.74±0.50**	99.45±0.28**	98.43±0.63**	98.42±0.38**
Ushirasav Low dose	2 mL/Kg	98.99±0.59**	101.45±0.44**	101.69±0.59**	100.14±0.49**	99.75±0.34**
Ushirasav High dose	4 mL/Kg	98.87±0.20**	101.50±0.34**	100.22±0.28**	99.98±0.61**	98.55±0.39***

#Results are expressed as mean±SD (n=6). *P<0.05; **P<0.01; ***P<0.001compared to control

The antipyretic efficiency of Ushirasav is comparable with standard drug paracetamol. As fever rise in all groups in 1 h up to 101°F but after 4 h high dose and Paracetamol treated animal shows normal temperature of 98.42 to 98.55°F

COX-2 inhibitory effects: Cyclooxygenase-2 (COX-2) is a key enzyme in the inflammatory process and is a prominent target in the development of nonsteroidal anti-inflammatory drugs (NSAIDs). Unlike its isoform COX-1, COX-2 is inducible and plays a significant role in various pathological conditions including cancer, arthritis, and cardiovascular diseases. The selective inhibition of COX-2 can provide therapeutic benefits with potentially reduced side effects compared to non-selective NSAIDs.

Table 3: Detailed description of Binding affinity, residual interactions of COX-2 (PDB- 5KIR) with Indomethacin (positive control) and Benzoic acid

Molecules under study	Binding affinity (kcal/mol)	Residual interactions at the binding pocket of PDB:5KIR	H-bonds
Indomethacin	-8.9	Ala-151, Arg-44, Arg-469, Asn-34, Asn-43, Cys-36, Cys-41, Cys-47, Gln-42, Gln-461, Glu-465, Gly-45, Gly-135, His-39, Leu-152, Met-48, Pro-40, Pro-153, Pro-154, Pro-156, Tyr-130, Tyr-136, Val-46	Cys-41(B), Arg-44(B)
Sample_UA (Benzoic acid)	-7.4	Ala-527, Arg-120, Arg-513, Gly-354, Gly-526, His-90, Leu-352, Met-522, Phe-518, Ser-353, Ser-530, Trp-387, Tyr-355, Val-349, Val-523	Ser-530(A)

#PDB=Protein data bank; 5KIR=The structure of Rofecoxib bound to COX-2

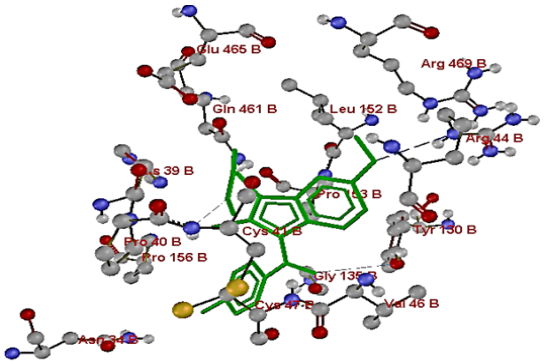

Fig. 2. Indomethacin docked at COX-2 (PDB- 5KIR)

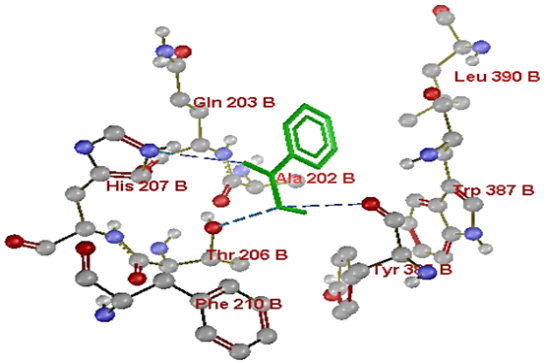

Fig. 3. Sample_UA(Benzoic acid) docked at COX-2 (PDB- 5KIR)

In this study, docking simulations revealed distinct interaction patterns of the compounds with COX-2, as detailed in PDB structure 5KIR. Indomethacin, serving as the positive control, exhibited a predominant interaction with chain B of COX-2, establishing hydrogen bonds with Cys-41(B) and Arg-44(B). This interaction suggests a robust binding mode that likely contributes to its well-documented efficacy as an anti-inflammatory agent.

In contrast, the query molecule SAMPLE-Ushirasav(Benzoic acid) was observed to engage with chain A of COX-2, forming a hydrogen bond with Ser-530(A). This indicates a different binding profile compared to indomethacin, potentially reflecting a unique mechanism of interaction with the enzyme.

These findings contribute to a nuanced understanding of the binding dynamics of these molecules with COX-2, highlighting the differential interactions and binding affinities that may influence their potential as therapeutic agents.

The results of molecular docking show binding affinity of Benzoic acid is comparable with indomethacin even on receptor COX-2 Benzoic acid has more binding affinity(docking score) than Indomethacin.

CONCLUSION

This study shows that fermented traditional formulation Ushirasav contains many bioactive compounds and these compounds have medicinal activity mainly related to inflammation. The results of in vivo studies also support that Ushirasav has antipyretic potential as compared to the standard drug Paracetamol and *in vivo* COX-2 inhibitory activity suggest the major constituent of Ushirasav (Benzoic acid) possess COX-2 inhibitory activity.So, it may be used to combat fever in various physiological conditions. In future Ushirasav may be used for treatment of pyrexia especially in pediatric and geriatric patients, who are more liable to liver disorders by Paracetamol.

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Conflict of Interest

"The authors do not have any conflict of interest."

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