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Analgesic and Anti-inflammatory Activities of *Trayodashang Guggulu*, an Ayurvedic Formulation

Shivani Gupta [a], Yadu Nandan Dey [b,c], Pushpendra Kannojia [a], Amit Kumar Halder [b], Deepti Sharma [c], Manish M. Wanjari [c,*], Shridhar Chougule [d], Sharad Pawar [d], Atul Kaushik [a], Sudesh N. Gaidhani [e], Shailendra Gurav [f]

[a] *Department of Pharmacology, Institute of Professional Studies College of Pharmacy, Gwalior, 474 002, Madhya Pradesh, India*
[b] *Dr. B.C. Roy College of Pharmacy and Allied Health Sciences, Durgapur, 713206, West Bengal, India*
[c] *Regional Ayurveda Research Institute for Drug Development, Gwalior, 474 009, Madhya Pradesh, India*
[d] *Regional Ayurveda Institute For Fundamental Research, Pune, Maharashtra, India*
[e] *Central Council for Research in Ayurvedic Sciences, New Delhi, 110 058, India*
[f] *Goa College of Pharmacy, Panjim, 403001, Goa, India*

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ABSTRACT

Background: *Trayodashang guggulu* (TG) is a compound Ayurvedic formulation used in Indian traditional system of medicine for the treatment of various inflammatory conditions like arthritis and associated pain.

Purpose:To study the analgesic and anti-inflammatory effects of *trayodashang guggulu*.

Methods: *Trayodashang guggulu* (TG) was standardized as per standard procedures and TLC profile was carried as per Ayurvedic Pharmacopoeia of India and LC-MS analysis was done to identify its bioactive constituents. *Trayodashang guggulu* was suspended in water and administered orally at 270 and 540 mg/kg dose for evaluating pain and inflammation in rats. Analgesic activity was assessed by Eddy's hot plate, tail immersion and formalin-induced pain models while anti-inflammatory activity was assessed by carrageenan and formalin-induced inflammation models. Further, the mechanism of anti-inflammatory action is predicted through various in silico methods like molecular docking and dynamics studies using AutodockVina and AMBER, respectively.

Results: TG was found compliant as per pharmacopoeial standards. TG (270 and 540 mg/kg, orally) did not cause significant reduction in pain in centrally acting pain models i.e. Eddy's hot plate and tail immersion tests as like Pentazocin (10 mg/kg). In formalin-induced pain model, TG (270 and 540 mg/kg, orally) significantly decreased both flinching and licking pain responses in early and late phase while indomethacin (10 mg/kg) only affected late phase flinching and licking. Further, TG showed significant time-dependent reduction in formalin and carrageenan-induced inflammation as compared to vehicle control. Indomethacin (10 mg/kg), standard drug also showed significant reduction in pain and inflammation. LC-MS analysis revealed the presence of 17 phytoconstituents in TG. Further, in silico studies revealed that some of the identified phytochemicals may have inhibitory activity against COX-2 enzyme and the synergistic effects due to multi components may be responsible for the anti-inflammatory properties of TG.

Conclusion: In conclusion, *trayodashang guggulu* inhibited the inflammatory pain as well as showed anti-inflammatory activity in rats. The effect may be attributed to the presence of anti-inflammatory phytoconstituents through the inhibition of anti-inflammatory enzymes like COX-2. The study further validates its traditional use in various painful inflammatory conditions.

Abbreviation: ANOVA, Analysis of variance; AqTG, Aqueous extract of *trayodashang guggulu*; COX2, Cyclooxygenase-2; TG, *Trayodashang guggulu*; TLC, Thin layer chromatography; NSAIDs, Non-steroidal anti-inflammatory drugs.

* Corresponding author.
E-mail address: manish.nriashrd@gmail.com (M.M. Wanjari).

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Introduction

Inflammation is biologically occurring reaction inside vascular tissues when exposed to any destructive stimuli (White, 1999). Like modern medicine, Ayurveda also signifies the process of inflammation as vascular and cellular reaction (Wasnik et al., 2017). Persistent and chronic inflammation leads to serious life threatening, painful and untreatable diseases like arthritis, gout, inflammatory bowel disease, asthma, tumours, etc. These inflammatory conditions are associated with intense pain typically called inflammatory pain (Nantel, 1999). Conventional modern medicines viz. non-steroidal anti-inflammatory drugs (aceclofenac, diclofenac etc), steroids (glucocorticoids) provide only symptomatic relief and do not provide satisfactory treatment. Their prolong use causes undesirable effects such as gastro-intestinal irritation, cardiovascular problems, drug dependency, thymus suppression, anemia, etc. (Fitz and Patrono, 2001; Warne et al., 2004).

In India, Ayurvedic traditional system of medicine advocates the use of herbs and mineral based medicines to manage pain and inflammatory conditions (Singh et al., 2008). The formulations are designed on the basis of Ayurvedic wisdom of pathogenesis of disease and *tridosha* (vata-pitta-kapha) theory of prakriti (constitution) of the diseased individual. There is growing interest among public towards Ayurvedic or herbal medicine for amelioration of such painful inflammatory conditions. Many single and compound Ayurvedic formulations are used successfully in clinical practice for treatment of inflammatory diseases (Rathore et al., 2007; Subramanyam et al., 2013).

Trayodashang guggulu is an important classical Ayurvedic polyherbal formulation. It is an official drug in Ayurvedic Formulary of India (Anonymous, 2003) and Ayurvedic Pharmacopoeia of India (Anonymous, 2008). It is described in Ayurvedic classical text *Bhaishaja Ratnavali* Shastri Ambikadatta, 2004 which mentions its traditional use in treatment of arthritis, lumbar-sacral and knee rigidity, sciatic pain, tempero-mandibular joint disorders, arm-pain, ligament injury and fractures. As the name trayodashang indicates, it contains 13 ingredients in addition to guggulu, viz. babula (stem bark of *Acacia nilotica* (L.) Delile of family Leguminosae), ashwagandha (roots of *Withania somnifera* (L.) Dunal of family Solanaceae), hapusa (fruits of *Juniperus communis* L. of family Cupressaceae), guduchi (stem of *Tinospora cordifolia* (Willd.) Miers of family Menispermaceae), shatavari (roots of *Aspargus recemosus* Willd. of the family Asparagaceae), gokshuara (fruits of *Tribulus terrestris* L. of family Zygophyllaceae), vradadaru (roots of *Argyreia nervosa* (Burm. f.) Bojer of family Convolvulaceae), rasana (roots and leaves of *Pluchea lanceolata* (DC.) C.B.Clarke of family Compositae), satavha (fruits of *Anethum sowa* Roxb. ex Fleming of the family Apiaceae), sati (rhizome of *Hedychium spicatum* Sm. of the family Zingiberaceae), yavani (fruits of *Trachyspermum ammi* (L.) Sprague of family Apiaceae), sunthi (rhizome of *Zingiber officinale* Roscoe of family Zingiberaceae), shuddha guggulu (exudates of *Commiphora wightii* (Arn.) Bhandari of family Burseraceae) and goghrat (ghee) (Anonymous, 2003; Anonymous, 2008). The various proportions of the ingredients are mentioned in Table S1 (Supplementary material). The phytoconstituents of most of its individual plants ingredients such as phytosterols, flavonoids, triterpenoids, total phenols, tannins etc. demonstrated anti-inflammatory activity in various experimental models (Jeet and Thakur, 2012; Battu and Kumar, 2010; Sokeng et al., 2013; Prasad et al., 1965; Chawla et al., 1991; Dixit and Tiwari, 1991; Gupta and Singh, 2014; Rosen et al., 2000; Ghildiyal et al., 2012; Penna et al., 2003). In clinical study, it exhibited beneficial action on osteoarthritic subjects (Biswal et al., 2015). Previously, we demonstrated antioxidant and anti-inflammatory effects of *trayodashang guggulu* in *in-vitro* models (Dadoriya et al., 2020).

Despite the traditional use of *trayodashang guggulu* in inflammatory diseases and associated pain, no systematic experimental studies were conducted to delineate its effect on inflammation and associated pain. Therefore, it was felt necessary to investigate analgesic and anti-inflammatory effect of *tryodashang guggulu* in experimental models of pain and inflammation to validate its traditional use.

Materials and methods

Chemicals and drugs

Quercetin and carrageenan were procured from Sigma Aldrich, USA. Indomethacin was procured as gift sample from Alfa Remedies, Ambala. Pentazocin (Fortwin®, Ranbaxy Fine Chemicals Ltd., India) injections were procured from local chemist shop. Formaldehyde (Fisher Scientific Pvt. Ltd) and tannic acid (Merck) were procured from scientific suppliers.

A quality compliant *trayodashang guggulu* manufactured by IMPCOPS, Chennai (Batch No. APT-156) was obtained from Drug store of our Institute.

Standardization of trayodashang guggulu

The standardization of *trayodashang guggulu* was carried out using standard procedures (Khandelwal, 2006).

Phytochemical studies

Trayodashang guggulu was powdered and maceration in distilled water for 48h to obtain aqueous extract (AqTG). Preliminary phytochemical screening of the formulation was done for qualitative detection of carbohydrates, flavonoids, phenolic compounds, steroids, saponins, tannins alkaloids, etc present in the AqTG (Khandelwal, 2006).

Total phenolic content (Singleton et al., 1999) and total flavonoid content (Marinova et al., 2005) were estimated spectrophotometrically.

Thin layer chromatography fingerprint analysis of Trayodashang guggulu

Thin layer chromatography (TLC) profile of *trayodashang guggulu* was evaluated by the method described in Ayurvedic Pharmacopoeia of India (Anonymous, 2008).Briefly, 5 g of *trayodashang guggulu* powder was subjected to extraction by refluxing in n-Hexane (75 ml) for 30 min. Then it was filtered and concentrated to 25 ml and was used for TLC study. Ten µl of the concentrate was applied on TLC plate and developed to 8 cm. The mobile phase was toluene:acetone (9:1). After development, plate was allowed to dry in air and examined in UV cabinet (at 366 nm). The derivatization of the plate was done using anisaldehyde-sulphuric acid reagent. The spots were identified and the respective R_f values were calculated.

LC–MS/MS analysis

LC–ESI–MS/MS analysis was carried out by using a UHD Accurate-Mass 6538 Q-TOF LCMS system (Agilent Technologies) with a Infinity Lab Poroshell 120SB-C18 analytical column (3.0 × 100 mm, 2.7 µm). Q-TOF system is state of the art platform for MRM analysis with less than 5 ppm error yielding very high mass resolution. The mobile phase was comprised of solvent A (H_2O: Acetonitrile: Formic acid; 90:9.9:0.1), solvent B (Acetonitrile: H_2O: Formic acid; 90:9.9:0.1) run in a gradient mode (5% to 97%). The injection volume was 20.00 µl and the column temperature was maintained at 40°C. Parameters for analysis were set using positive ion mode with spectra acquired over a mass range from m/z 100 to 1700 for MS and from m/z 50 to 1700 for MS/SM, data was acquired at 2 GHz extended dynamic range with narrow isolation width. The MS/MS data was analyzed using quantitative analysis software (Version B.10.0 Agilent Technologies, USA), the compounds were identified using commercially available licensed METLIN metabolite PCDL library. The accuracy for confirmation of the compounds was established on the basis of their error less than 5 ppm and MS/MS fragment matching.

Animals

Male Wistar rats (250-300 g) were housed at temperature (22±3°C); relative humidity (50±5%) and 12 h (light: dark) cycle. Rats were fed rodent chow (Ashirwad brand, Chandigarh, India) and water *ad libitum*. Ethical guidelines were followed to execute the protocol after its approval by ethical committee (Proposal No. NRIASHRD-GWL/IAEC/2014/4).

Justification of dose selection

Oral dose of *trayodashang guggulu* from human equivalent dose was calculated by using conversion formula mentioned in standard textbook (Paget and Barnes, 1964). The calculated therapeutic dose is 270 mg/kg body weight for rats. Based on this, the doses of 270 and 540 mg/kg were selected for the study. In Ayurvedic text, the vehicle (*anupana*) for *trayodashang guggulu* is water or milk (Ayurvedic Pharmacopoeia of India, 2008). Hence, a suspension of *trayodashang guggulu* powder in deionized water containing 2% gum acacia was prepared and fed to rats by feeding cannula.

Acute toxicity study

Acute oral toxicity study was performed by following the procedures mentioned in OECD 423 guidelines (OECD, 2001) and previous study (Dey et al., 2017). The rats were divided into two groups (n=3). Control group received 2% gum acacia (prepared in deionized water) as vehicle at a dose volume of 5 mL/kg body weight while the test treated group was orally administered *Trayodashang guggulu* in the limit test dose of 2000 mg/kg. The rats were observed continuously for behavioral, neurological and autonomic profiles for 2 h and after a period of 24, 72 h and thereafter up to 14 days for any lethality, moribund state or death. Cage side observations included change in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behavior pattern. All the animals were observed once daily for morbidity and mortality. The limit test was repeated in another groups of rats (n=3) for confirmation and toxic class of LD_{50} determination. Animals were euthanized by CO_2asphyxia on 15th day of the study and subjected to detailed post-mortem examination.

Pharmacological investigations

In each study, the rats were divided into 4 groups each containing 6.

Group I: Vehicle control; administered 2% gum acacia

Group II: Reference standards (Pentazocin or indomethacin as per biological activity)

Group III -IV: *Trayodashang guggulu* (270 mg/kg and 540 mg/kg, orally, respectively)

Evaluation of analgesic activity

Eddy's hot plate and tail immersion models

The analgesic effect of TG on centrally mediated pain was evaluated in rats as per the previously described methods i.e. Eddy's hot plate (Eddy and Leimbach, 1953) and tail immersion (Upudha et al., 2007) methods at 55 ± 1°C. After the treatment with test drug and pentazocin (10 mg/kg, intraperitoneally) the reaction time (in sec) of the rats at 30 min and 60 min of drug administration was recorded. The percentage increment of reaction time was calculated by the following formula.

$$\%\text{Increaseinreactiontime} = \frac{[\text{Rt} - \text{Rb}]}{[\text{Rb}]} \times 100$$

Where, Rt = Reaction time of treated drug, Rb = Basal reaction time

Formalin-induced pain

The effect of TG on inflammatory pain was assessed by formalin-induced pain in Wistar rats (Dubuisson and Dennis, 1977). After 1 h treatments in respective groups, 0.05ml of 2.5 % commercially available 37% formalin was injected into the left hind paw of and observed for 30 min. After formalin injection, the early phase (first 10 min) and the late phase (between 10 and 30 min) represents both neurogenic and inflammatory pain response, respectively. The numbers of licking and flinching were counted in both phases during the period. Indomethacin (10 mg/ kg, orally) was used as a standard.

Evaluation of anti-inflammatory activity

Anti-inflammatory effect was assessed by the procedure mentioned in previous studies using two different models i.e. formalin-induced and carrageenan-induced paw edema (Pandey et al., 2017) in rats. In each model, after 1 h of drug administration, 0.05ml of 2.5 % formalin or 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline was injected into the left hind paw of each rat. The paw edema was measured in ml using plethysmometer (UGO Basile, Italy) at 0, 1, 3 and 5 h after formalin or carrageenan administration. Indomethacin (10 mg/kg) was used as a standard.

The percent inhibition was calculated by following formula

$$\text{Percentageinhibition} = \frac{(\text{Pv}_t - \text{Pv}_o)\text{control} - (\text{Pv}_t - \text{Pv}_o)\text{treated}}{(\text{Pv}_t - \text{Pv}_o)\text{control}} \times 100$$

Where, Pv_t = Paw volume after formalin or carrageenan injection and

Pv_o = Paw volume before formalin or carrageenan injection

In-silico studies

Similarity search analysis

The similarity search analysis was performed using newly developed open-access tool named SIMSEARCH (https://github.com/ncordeirfcup/SIMSEARCH), which calculates the Tanimoto similarity between query and target compounds based on various fingerprints. In the present work, extended-connectivity fingerprints with up to four bonds (ECFP4), were used to obtain structurally similar query compounds with respect to the target compounds (Bajusz et al., 2020; Capecchi et al., 2020; Halder et al., 2021).

In the current investigation, an attempt was made to understand if any of the phytochemicals identified by LC-MS analysis of *trayodasang guggulu* (Table 2; Fig. 2) is responsible for the inhibition of COX-2 enzyme, which is one of the most common targets for anti-inflammatory properties. *In silico* methods were used to predict the role of phytoconstituents in COX-2 enzyme through the prediction of such properties for mixture of phytochemicals are not straightforward. It is to remember that synergistic effects may exist and anti-inflammatory property obtained here may have resulted due to combined effects of phytochemicals with weak to moderate potencies against COX-2. Furthermore, there are chances of *in vivo* transformation of some of these phytochemicals. Nevertheless, the attempt was made to understand the role of each of these phytochemicals (with molecular weight less than 1000) against the COX-2 enzymes. The main objective was to apply structure-based modelling approaches to check if any of these chemicals have potential to bind at the active site of COX-2 enzyme. However, due to structural natures, the docking of the several phytochemicals may have more complications as compared to small molecule inhibitors. Therefore, special attention needs to be given to the phytochemicals for which theoretical druggability properties are not satisfied. Initially druggability properties of 17 phytochemicals with molecular weight less than 1000 was studied. The SwissADME server (http://www.swissadme.ch/) (Daina et al., 2017) was implemented to calculate

the druggability of these chemicals with respect to Lipinsky rule (Lipinski et al., 1997), Ghose rule (Ghose et al., 1998), Veber rule (Veber et al., 2002), Egar rule (Egan et al., 2000) and Muegge rule (Muegge et al., 2001). The total numbers of violations of these rules were summed to estimate overall druggability of these 17 phytochemicals.

Fortunately, COX-2 is one of the most investigated bio-macromolecular targets and therefore its protein complexes (with bound ligands) are available clearly defining the active site of this enzyme and binding modes of the ligands. Similarly, several compounds have been investigated with COX-2 inhibitory potentials. Therefore, apart from molecular docking we relied on relatively simpler method such as fingerprint-based similarity search analyses to understand the potential of these phytochemicals to inhibit the COX-2 enzyme. For this purpose, a newly developed publicly-available tool named SIMSEARCH (https://github.com/ncordeirfcup/SIMSEARCH) was used which is Python based tool that utilizes various fingerprints calculated with well-known RDkit program to check structural similarities among a group of query compounds against a set target compounds. For example, in this work we submitted these 17 phytochemicals as query ligands. Similarity dataset of 10,933 chemicals were collected from ChEMBL with inhibitory properties against COX-2 enzyme and treated as target database. For this work, the data with 3 experimental endpoints namely IC_{50}, EC_{50} and percentage of inhibition were considered. A cut-off value of 0.45 was considered and the query database compounds exhibiting similarity greater than 0.45 with at least one target database compound were analyzed. The hits from target database were then queried for their reported inhibitory potentials against COX-2 in ChEMBL database.

Molecular docking analysis

The X-ray crystal structures of COX-2 [PDB ID: 5IKR (Orlando and Malkowski, 2016)] were retrieved from the Protein Data Bank. The compounds were first docked at the catalytic site of these enzymes defined by the location of the small molecule inhibitors complexed with these proteins, using the AutodockVina v1.2.0 (Trott and Olson, 2009). A grid size of 40 Å × 40 Å × 40 Å was defined from the bound ligands located at the catalytic site of the proteins. The 2D ligand-receptor interactions were obtained from PoseView (Stierand K et al., 2006) using the webserver ProteinsPlus (https://proteins.plus/).

Molecular dynamics simulations

The protonation states of amino acid residues of the protein complexes were fixed at pH = 7.0 by the PDB2PQR server (http:// server.poissonboltzmann.org/pdb2pqr), using the AMBER forcefield and output naming scheme (Dolinsky et al., 2007). The ff99SB and the general AMBER force field (GAFF) were employed for describing receptor-ligand and ligand–water interactions, respectively (Wang et al., 2004). The remaining methods for 50 ns simulation, trajectory analyses and Molecular Mechanics Generalized Born Surface Area (MM-GBSA) (Srinivasan et al., 1998; Ylilauri and Pentikäinen, 2013) based binding free energies have been described previously (Halder and Honarparvar, 2019; Halder et al., 2021) and therefore are not being described here.

Statistical analysis

Results were analyzed by one-way and two-way ANOVA followed by Tukey's and Bonferroni post hoc test, respectively, wherever necessary. $P < 0.05$ was considered significant in all cases

Results

Phytochemical studies

The observation of physiochemical/standardization parameters for *trayodashang guggulu* is shown in Table 1. The preliminary phytochemical screening revealed that *trayodashang guggulu* showed the presence of phytosterols, phenolics, saponins, tannins. The quantitative estimation showed 83.48 mg quercetin equivalent/g of powdered drug as total flavonoids while 40.62 mg tannic acid equivalent/g of powdered drug as total phenolic content.

Table 1
Physicochemical evaluation of *Trayodashang guggulu*

Standardization parameters	Value	Pharmacopoeial Limits
Ash analysis		
Ash content (Total Ash) (%w/w)	9.64±0.07	NMT 15%
Acid In-soluble ash (%w/w)	2.61±0.06	NMT 4%
Water soluble ash (%w/w)	7.01±0.06	——
Extractive value (Maceration Process)		
Alcohol soluble	24.81±0.86	NLT 17.5%
Water soluble	28.43±1.13	NLT 21%
Moisture content (Loss On Drying)	4.72±0.04	NMT 11%
pH (1% aqueous solution)	4.77±0.01	4.45-5.96

Values are expressed as mean±SEM (n=3).
NMT.-Not more than; NLT- Not less than.

TLC fingerprint of trayodashang guggulu

TLC plate showed 4 spots at R_f 0.19, 0.37, 0.44 and 0.59 (all fluorescent blue). After derivatisation, the plate showed2 spots at R_f0.40 and 0.61 (all pink changing to purple) in visible light. The TLC photographs are depicted in Fig. 1.

Metabolite profiling of trayodashang guggulu

The LC-MS chromatogram of methanolic extract *trayodashang guggulu* is represented in Fig.2. Results from LC-MS analysis revealed the identification of 17 phytoconstituents in extract after integrating with the libraries. The details of the compounds are mentioned in [Table 2].

Acute toxicity study

In acute toxicity study, limit test dose of 2000 mg/kg of the TG did not cause death of rats during 14 days observation period. The rats did not show any signs of toxicity or change in general behavior compared to vehicle control group. No lethality or any toxic reactions or moribund state were observed up to the end of the study period. There were no significant changes observed in weekly body weights of rats treated with TG at 2000 mg/kg when compared to vehicle control (Table 3). During necropsy, no gross morphological changes were observed in the internal organs from test groups compared to vehicle control rats. The approximate LD_{50} of TG is greater than 2000 mg/kg.

Analgesic activity of *trayodashang guggulu*

Effect on pain response in hot plate test

Treatment with TG (270 and 540 mg/kg) did not show any significant change in reaction time at 30 and 60 min of treatment as compared to basal reaction time of vehicle control rats (Table 4). On the contrary, pentazocin treatment significantly ($p<0.01$) increased the reaction time of the animals at 60 min compared to vehicle control without any effect at 30 min. The percentage increase in reaction time by pentazocin (10 mg/kg) at 60 min was 7%.

Effect on pain response in tail immersion test

Treatment with TG (270 and 540 mg/kg)did not show any significant change in reaction time at 30 and 60 min of administration as compared to vehicle control rats (Table 5). Pentazocin treatment significantly increased the reaction time at 30 min ($p<0.05$) and 60 min ($p<0.001$) as compared to vehicle control. The percent increment of reaction time by

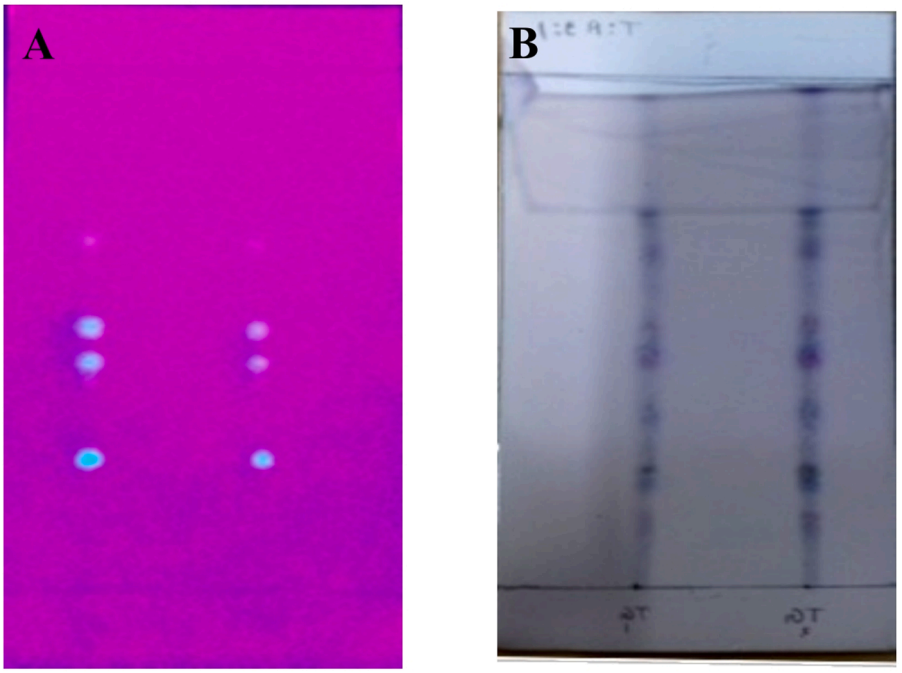


Fig. 1. TLC Profile of *Trayodashangguggulu*

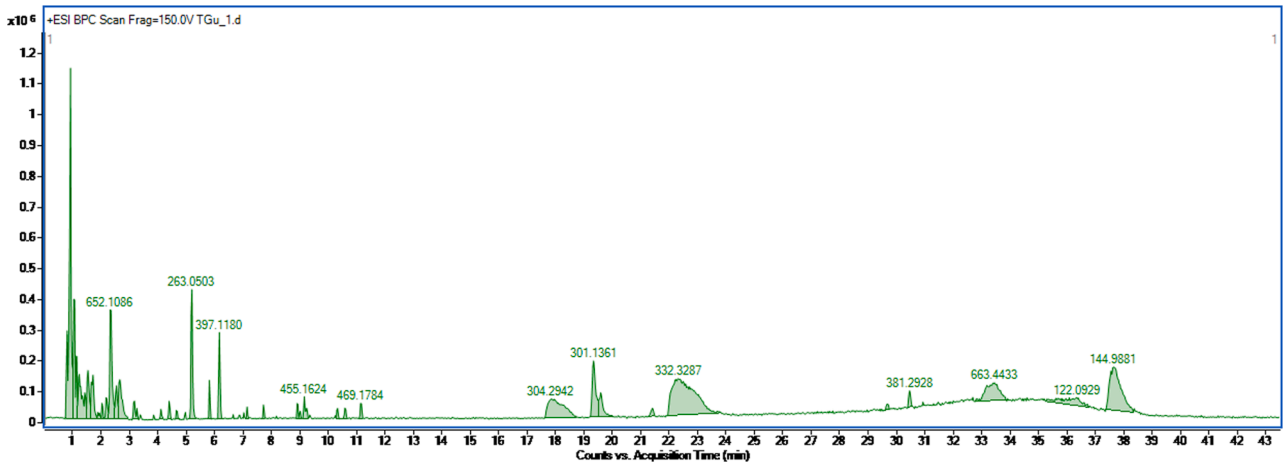


Fig. 2. A base peak chromatogram of *Trayodashang guggulu* (TGu) 100 µg concentration (20 µl of 5 mg/ml solution).

pentazocin at 30 and 60 min were 58% and 98%, respectively.

Effect on formalin-induced pain response

Effect on flinching response

Treatment with TG (270 and 540 mg/kg) significantly inhibited the early phase flinching ($p<0.001$) (Fig. 3A) while TG (270 and 540 mg/kg) and indomethacin treatment both inhibited late phase flinching ($p<0.05$ to $p<0.001$, wherever applicable) as compared to vehicle control group (Fig. 3B).

Effect on licking response

Treatment with TG (270 and 540 mg/kg) significantly ($p<0.01$) inhibited the early phase licking as compared to vehicle treated rats (Fig. 3C) while TG (270 and 540 mg/kg) and indomethacin significantly ($p<0.05$ to $p<0.001$, wherever applicable) reduced the late phase licking as compared to vehicle control rats (Fig. 3D).

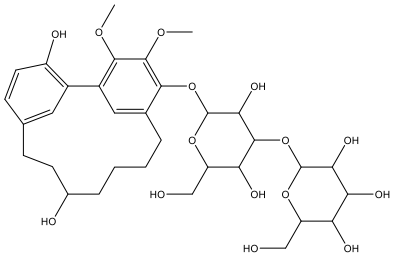
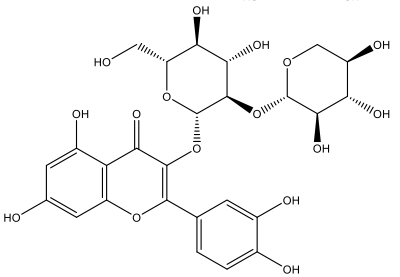
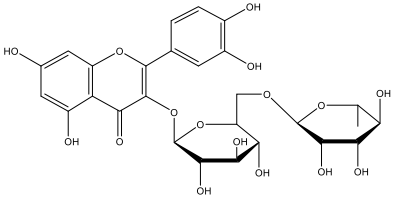
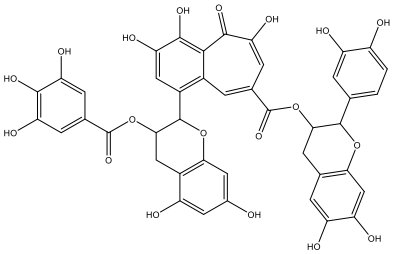
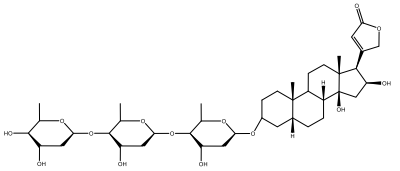
Effect on inflammatory response

Effect on formalin-induced paw edema

Treatment with TG (270 and 540 mg/kg) and indomethacin significantly ($p<0.05$ to $p<0.001$, wherever applicable) inhibited the formalin-induced increase in paw volume at 1, 2 and 5 h of formalin administration as compared to vehicle control rats (Table 6).

The percentage inhibition of increase in paw volume at 1st, 3rd and 5th h was 30.95, 33.33 and 36.11 by TG (270 mg/kg) and 35.71, 33.33

Table 2
LC-MS-MS analysis of *Trayodasang guggulu*

S. No.	Name of the compound	METLIN	Retention time	m/z	Charge state	Mass	Chemical formula	Abundance	Error PPM (<5ppm)	Structure
1	Myricanol 5-laminaribioside	91623	1.248	342.1495	2	682.2856	C33 H46 O15	3733	2.84	
2	Quercetin 3-sambubioside	50506	3.421	299.0764	2	596.1382	C26 H28 O16	543	0.71	
3	Rutin	3677	3.604	611.1562	1	610.1489	C27 H30 O16	5480	-4.45	
4	Theaflavate A	88962	3.773	449.0677	2	852.1497	C43 H32 O19	847	-4.75	
5	Gitoxin	57786	4.866	408.2494	2	780.4281	C41 H64 O14	589	-1.97	

(*continued on next page*)

Table 2 (*continued*)

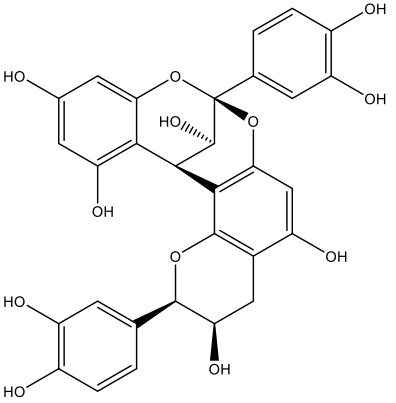
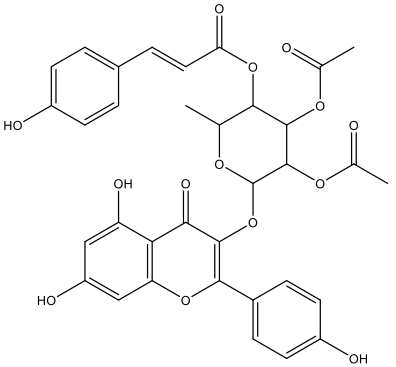
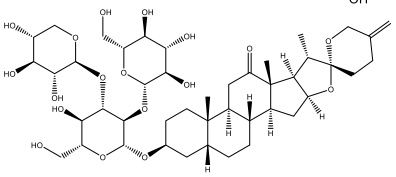
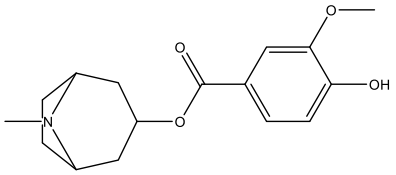
6	Proanthocyanidin A2	68190	4.982	311.0538	2	576.1275	C30 H24 O12	1129	1.26	
7	Kaempferol 3-(2′′,3′′-diacetyl-4′′-p-coumaroylrhamnoside)	95028	5.28	332.089	2	662.1634	C34 H30 O14	966	-0.24	
8	Schidigerasaponin B1	90788	5.865	443.2292	2	884.4393	C44 H68 O18	1125	-1.39	
9	Phyllalbine	68639	6.04	314.1352	1	291.1461	C16 H21 N O4	2122	-3.16	

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Table 2 (*continued*)

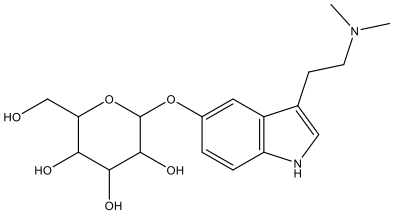
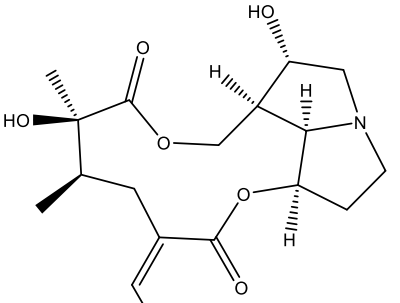
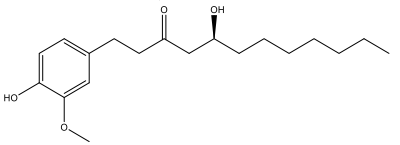
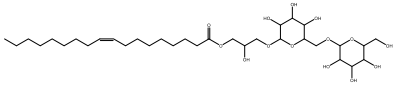
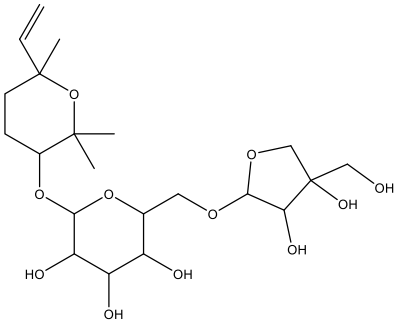
10	Bufotenine O-glucoside	86346	6.297	367.1853	1	366.178	C18 H26 N2 O6	9445	-3.04	
11	Rosmarinine	68291	7.762	376.172	1	353.1833	C18 H27 N O6	8581	-1.66	
12	(8)-Gingerol	71735	8.503	345.2027	1	322.2136	C19 H30 O4	3319	-2.57	
13	Gingerglycolipid C	95528	9.56	363.1882	2	680.3968	C33 H60 O14	947	-2.17	
14	Linalool oxide D 3-[apiosyl-(1->6)-glucoside]	87620	10.611	233.1193	2	464.2252	C21 H36 O11	882	-1.21	

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Table 2 (*continued*)

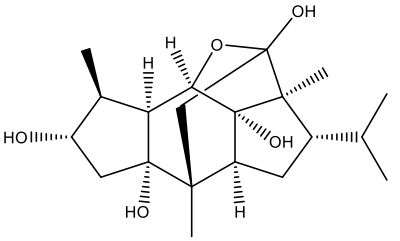
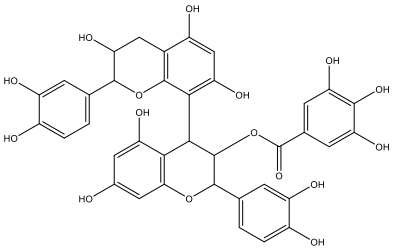
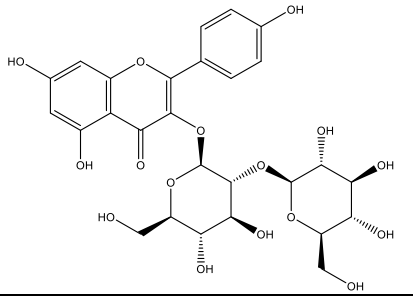
15	Cinncassiol D4	71844	10.814	375.2139	1	352.2259	C20 H32 O5	545	2.56	
16	ent-Epicatechin-(4alpha->8)-ent-epicatechin 3-gallate	92754	14.861	383.1122	2	730.1519	C37 H30 O16	475	-1.97	
17	Kaempferol 3-O-Î²-D-glucosyl-(1->2)-Î²-D-glucoside	64229	26.113	633.1433	1	610.153	C27 H30 O16	2168	-0.59	

Table 3
Effect of acute dose of *trayodasang guggulu* on clinical signs and body weight

Observations of toxic signs in following parameters	Control (vehicle)	TG 2000 mg/kg
Skin and Fur	Normal	No change
Eyes and mucus membrane	Normal	No change
Respiratory system	Normal	No change
Circulatory system	Normal	No change
Autonomic nervous system	Normal	No change
Central nervous system	Normal	No change
Somatomotor activity	Normal	No change
Behavioral pattern	Normal	No change
Tremor	Normal	No change
Convulsions	Normal	No change
Salivation	Normal	No change
Diarrhea	Normal	No change
Lethargy	Normal	No change
Sleep	Normal	No change
Coma	Normal	No change
Body weight		
Day 1	183.33±2.58	185.50±2.26
Day 7	193.83±2.64	196.17±3.43
Day 14	205.83±2.64	207.50±2.43

Body weights are expressed in grams. Values are expressed in mean±SEM, n=6. No significant differences were observed among the groups. [TG: *Trayodashang guggulu]*

Table 4
Effects on Eddy's hot plate test

Groups	Dose (mg/kg)	Basal reaction time (sec)	Reaction time (sec)	
		0 min	30 min	60 min
Vehicle Control	-	1.83±0.10	1.83±0.10	1.75±0.11
Pentazocin	10	2.41±0.20	2.58±0.32	2.91 ±0.42@
TG	270	2.25±0.25	2.65±0.11	2.50±0.22
	540	2.25±0.22	2.25±0.18	2.48±0.31

Results were expressed as mean±SEM (n=6)
@P<0.01 when compared to vehicle control
[TG= *Trayodashang guggulu*]

Table 5
Effect on tail immersion test

Groups	Dose (mg/kg)	Basal reaction time (sec)	Reaction time (sec)	
		0 min	30 min	60 min
Vehicle Control	-	4.36±0.63	4.2±0.67	4.25 ±0.73
Pentazocine	10	4.33±0.76	6.87±0.54$	8.58 ±1.52#
TG	270	4.5±0.22	5.08±0.27	5.5±0.22
	540	4.92±0.52	5.33±0.61	6.33 ±0.42

Results are expressed as mean±SEM (n=6)
$P<0.05, #P<0.001 when compared to vehicle control
[TG= *Trayodashang guggulu*]

and 47.22 by TG (540 mg/kg), respectively. The percentage inhibition of increase in paw volume by indomethacin at 1st, 3rd and 5th h was 61.9, 56.41and 69.44, respectively.

Effect on carrageenan-induced paw edema

Treatment with TG (270 and 540 mg/kg) significantly decreased the paw volume at 3rd ($p<0.05$ and $p<0.01$, respectively) and 5th h ($p<0.01$ and $p<0.001$, respectively) as compared to vehicle control rats without any significant change at 1st h. Indomethacin treatment also significantly ($p<0.001$) decreased the paw volume at 3rd and 5th h without any significant effect at 1st h (Table 7).

The percentage inhibition of increase in paw volume by TG at 270 mg/kg at 3rd h and 5th h were 25.39 and 35, respectively while that of 540 mg/kg at 1st, 3rd and 5th h were 7.6, 30.15 and 41.25, respectively. The percentage inhibition of increase in paw volume by indomethacin at 10 mg/kg at 1st, 3rd and 5th h was 26.92, 13.88 and 61.25, respectively.

Similarity search analysis

It was observed that out of 17 compounds, 11 compounds have more than 10 violations, 1 compound (i.e., Linalool oxide-D-3-[apiosyl-(1->6)-glucoside]) has 8 violations and remaining 6 compounds have violations 0 or 1. Phyllabine was found to be the only compound which has zero violations and it should be considered as the most druggable phytochemical among others. The results are presented in Table 8.

The data of inhibitory potentials against COX-2 are represented in Table 9. As it is observed from Table 9, eleven phytochemicals depicted similarity greater than 0.45 with at least one target database compound and this table lists ChEMBL database compound that depicted maximum similarity with them. One of the most promising aspects of such similarity search analysis is that it retrieves the information from large databases very quickly. This analysis revealed that rutin has already been investigated for COX-2 inhibitory potential and it was proved to be a weak inhibitor of COX-2. One of the closest analogues of (8)-Gingerol is CHEMBL402978 [or (6) -gingerol] and it was also reported to have IC_{50} of 125 μM against COX-2 enzyme. Therefore, there is a possibility that (8)-gingerol may be a potential COX-2 inhibitor with weak to moderate activity. On the other hand, compounds with high structural similarity with Gitoxin and Quercetin-3-sambubioside were found to be inactive against COX-2. One structurally similar compound of Proanthocyanidin A2 depicted 44% inhibition of COX-2 enzyme at 100 μg/ml. Similarly, IC_{50} of 125 μM was obtained from a compound that depicted structural similarity of 0.64 with ent-Epicatechin-(4alpha->8)-ent-epicatechin 3-gallate. Unlike Proanthocyanidin A2 and ent-Epicatechin-(4alpha->8)-ent-epicatechin 3-gallate, that are structurally large and have low theoretical druggability properties, phyllabine is structurally small and has high theoretical druggability properties. Even though a structurally similar analogue of phyllabine (i.e., CHEMBL56564) failed to show any activity against COX-2, we relied mainly on its structure-based prediction (molecular docking and molecular dynamics simulations that is discussed later). The last four phytochemicals were matched with the target database with moderate structural similarity but none of these found to have potential COX-2 inhibitory properties. However, it is noteworthy that the similarity analyses were performed just to understand overall scopes of these phytochemicals for being COX-2 inhibitors and it is well known that even minor modifications in the structure may also lead to considerable changes in the biological activity. Moreover, some of these phytochemicals may be chemically transformed *in vivo* to exhibit COX-2 inhibitory property. For example, even though Rutin was found to be less active, quercetin (CHEMBL50) has been reported with IC_{50} value of 28.6 μM against COX-2. Same logic is applicable to Quercetin 3-sambubioside.

Molecular docking analysis

The molecular docking of the 17 phytochemicals was done with X-ray crystal (PDB: 5IKR) structure of the COX-2 enzyme. The docking method was first validated with self-docking where the bound ligand mefenamic acid was docked at the active site of the COX-2 enzyme and the best pose was generated with docking score of -9.5 kcal/mol as well as an RMSD of 0.56Å as compared to the bound ligand. After validation of docking protocol, each of 17 phytochemicals were subsequently docked at the binding site using the same docking protocol.

The docking results are depicted in Table 10. It was observed that except (8)-gingerol and phyllabine, all these phytochemicals including rutin failed to bind at the active site of the enzyme and their docking poses were obtained outside the entrance of this binding site. For these

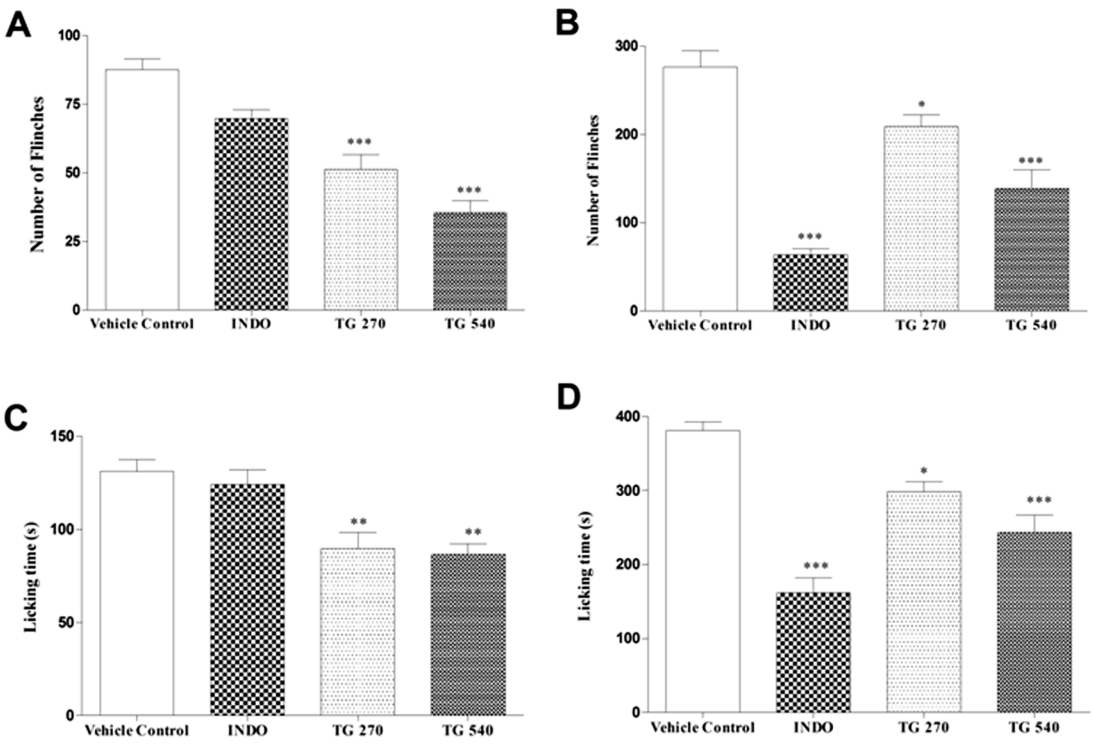


Fig. 3. Effect of *Trayodashang guggulu* on formalin-induced pain. 3A- Effect on early phase flinching; Fig. 3B- Effect on late phase flinching; Fig. 3C- Effect on early phase licking; Fig. 3D- Effect on late phase licking. Results are expressed as mean±SEM (n=6); Doses are expressed as mg/kg. *P<0.05; **P<0.01; ***P<0.001 when compared to vehicle control. [TG- *Trayodashang guggulu*, INDO- indomethacin]

Table 6
Effect on formalin-induced paw edema

Groups	Dose (mg. kg)	Percentage increase in paw volume at different time interval after formalin injection		
		1h	3h	5h
Vehicle Control	-	0.42±0.05	0.39 ±0.03	0.36 ±0.04
Indomethacin	10	0.16±0.03#	0.17 ±0.04#	0.11 ±0.04#
TG	270	0.29±0.03$	0.26 ±0.01$	0.23 ±0.02$
	540	0.27±0.02@	0.26 ±0.03$	0.19 ±0.05@

Results are expressed as mean±SEM (n=6)
$P<0.05; @P<0.01; #P<0.001 when compared to vehicle control,
[TG = *Trayodashang guggulu*]

Table 7
Effect on carrageenan-induced paw edema

Groups	Dose (mg/ kg)	Percentage increase in paw volume at different time interval after carrageenan injection		
		1h	3h	5h
Vehicle Control	-	0.26±0.01	0.63 ±0.02	0.80 ±0.04
Indomethacin	10	0.19±0.03	0.31 ±0.04#	0.31 ±0.06#
TG	270	0.26±0.01	0.47 ±.05$	0.52 ±.05#
	540	0.24±0.03	0.44 ±0.04@	0.47 ±0.05#

Results are expressed as mean±SEM (n=6)
$P<0.05; @P<0.01; #P<0.001 when compared to vehicle control
[TG= *Trayodashang guggulu*]

15 phytochemicals (which were docked outside active site), the docked pose of rutin (shown in Fig. S1, supplementary materials) may serve as a reference to discuss since its docked poses were located at the same position where these phytochemicals were found to dock. Note that rutin was also docked with high score but interacted with amino acids that form the entrance of the active site (e.g., Gly354) and outer shell of the side pocket (e.g. Gln192) (Moussa et al., 2021). Being a weak inhibitor, the docking of rutin may suggest that the structurally large phytochemicals that are docked at the same position may also exhibit moderate, weak or no inhibition of COX-2, at least in their non-transformed forms. Only (8)-gingerol and phyllabine were successfully docked inside the binding pocket but the docking scores are considerably less than mefenamic acid. The docked poses of these two compounds are depicted in Fig. 4. (8)-gingerol forms hydrogen bond interactions with Tyr355 and Tyr385 as well as Arg120 whereas its aromatic ring, which was superimposed with the one aromatic ring of mefenamic acid may form hydrophobic interactions with Ala527, Leu352, Val523, Val116, Leu93, etc. Similarly, phyllabine forms hydrogen bond interactions with the Tyr385. Note that the bound structure of mefenamic acid was also found to form hydrogen bond with this residue. Apart from making hydrogen bond interaction with this residue, phyllabine was found to form hydrophobic interactions mainly with Leu531, Val349 and Ala527.

Molecular dynamics simulations

The docked complexes of (8)-gingerol and phyllabine as well as bound complex of mefenamic acid with COX-2 enzyme were then subjected to 50ns explicit solvent MD simulations to understand their dynamic behaviors. The trajectory analyses (depicted in Fig. 5) led to the RMSD plots of protein complexes and ligands . Note that all these complexes were stabilized after 20ns. As far as the ligands are concerned, it is found that phyllabine displayed similar fluctuations as mefenamic acid in the 50ns run and the dynamic stability of these two

Table 8
Overall drug-likeness of the phytochemicals based number of violations

Phytoconstituent	Violations					Drug likeness
	Lipinski	Ghose	Veber	Egan	Muegge	
SchidigerasaponinB1	3	4	1	1	5	14
Gitoxin	3	3	1	1	5	13
ent-Epicatechin-(4alpha->8)-ent-epicatechin 3-gallate	3	3	1	1	4	12
Myricanol-5-laminaribioside	3	4	1	1	4	13
Gingerglycolipid C	3	3	2	1	5	14
Kaempferol 3-(2'',3''-diacetyl-4''-p-coumaroylrhamnoside)	2	3	2	1	3	11
Kaempferol 3-O-Î²-D-glucosyl-(1->2)-Î²-D-glucoside	3	4	1	1	4	13
Rutin	3	4	1	1	4	13
Quercetin-3-sambubioside	3	3	1	1	3	11
Proanthocyanidin A2	3	2	1	1	3	10
Theaflavine A	3	2	1	1	3	10
Linalool oxide D 3-[apiosyl-(1->6)-glucoside]	2	1	1	1	3	8
Bufotenine-O-glucoside	0	1	0	0	0	1
Rosmarinine	0	0	0	0	0	0
Cinncassiol D4	0	0	0	0	0	0
(8)-Gingerol	0	0	1	0	0	1
Phyllalbine	0	0	0	0	0	0

Table 9
Results of similarity search analyses with experimental data collected from ChEMBL

Phytoconstituent	Similarity with	Similarity	Results
Rutin	CHEMBL226335	1.00	<30% inhibition at 100 uM
(8)-Gingerol	CHEMBL402978	0.91	IC50 of 125 uM
Gitoxin	CHEMBL254219	0.87	Not active
Quercetin 3-sambubioside	CHEMBL250450	0.75	Not active
Proanthocyanidin A2	CHEMBL1213876	0.67	44% inhibition of COX-2 at 100 ug/ml
ent-Epicatechin-(4alpha->8)-ent-epicatechin 3-gallate	CHEMBL447023	0.64	IC_{50} of 125 uM
Phyllalbine	CHEMBL56564	0.52	Not active
Bufotenine O-glucoside	CHEMBL232202	0.51	<30% inhibition at 100 uM
Gingerglycolipid C	CHEMBL428593	0.51	24.7% inhibition at 50 uM
Schidigerasaponin B1	CHEMBL404811	0.50	Not active
Theaflavate A	CHEMBL503797	0.50	37% inhibition of COX-2 at 170 uM

Table 10
Results of molecular docking analysis

Phytoconstituent	Autodock Vina docking score	Position
Myricanol-5-laminaribioside	-8.5	Outside active site
Quercetin-3-sambubioside	-8.0	Outside active site
Rutin	-9.2	Outside active site
Theaflavine A	-9.0	Outside active site
Gitoxin	-8.8	Outside active site
Proanthocyanidin A2	-9.3	Outside active site
Kaempferol 3-(2'',3''-diacetyl-4''-p-coumaroylrhamnoside)	-7.6	Outside active site
Schidigerasaponin B1	-9.4	Outside active site
Phyllalbine	-7.3	Inside active site
Bufotenine-O-glucoside	-7.9	Outside active site
Rosmarinine	-6.9	Outside active site
(8)-Gingerol	-7.2	Inside active site
Gingerglycolipid C	-7.2	Outside active site
Linalooloxide	-8.1	Outside active site
Cinncassiol D4	-7.7	Outside active site
ent-Epicatechin-(4alpha->8)-ent-epicatechin 3-gallate	-8.8	Outside active site
Kaempferol 3-O-Î²-D-glucosyl-(1->2)-Î²-D-glucoside	-8.2	Outside active site
Mefenamic acid	-9.5	Inside active site

ligands are considerably higher than that of (8)-gingerol. The higher fluctuation of the latter ligand should have occurred due to higher number rotational bonds in the side chain of its structure. The plots of radiation of gyrations however depicted satisfactory compactness of all these complexes.

Finally, we determined the theoretical binding energies ($\Delta G_{bind}(T)$) of these three compounds with the help of MM-GBSA analyses and these results are presented in Table 11. It is clearly observed that high enthalpic binding energies were obtained from all three ligands especially for (8)-Gingerol. However, owing to high entropic contributions its overall theoretical $\Delta G_{bind}(T)$ was reduced to -23.21 kCal/mol that is lower than mefenamic acid. The high entropy of (8)-Gingerol should have resulted from high fluctuations of this ligand (See Fig. 5B). Phyllabine, on the other hand, ended up with -20.10 kCal/mol. From the trajectory analyses and $\Delta G_{bind}(T)$ values it can be inferred that (8)-gingerol and phyllabine may exhibit moderate activity against COX-2 enzyme.

Discussion

In view of the traditional use of *trayodoshang guggulu* (TG) in Ayurvedic system of medicine for treatment of various inflammatory disease conditions, the present study was conducted to validate its traditional claim using experimental models. The effect of TG on nociceptive response was evaluated by hot plate method, tail immersion test and formalin-induced persistent pain model in rats while the anti-inflammatory effect was assessed by formalin and carrageenan-induced inflammation models in rats. Results suggested that TG possesses antinociceptive and anti-inflammatory properties.

The assessment of physiochemical parameters indicated that *trayodoshang guggulu* intended for study was of requisite pharmacopoeial standard (Table 1). The TLC fingerprint and R_f values were also found similar as stated in Ayurvedic Pharmacopoeia of India (Anonymous, 2008).

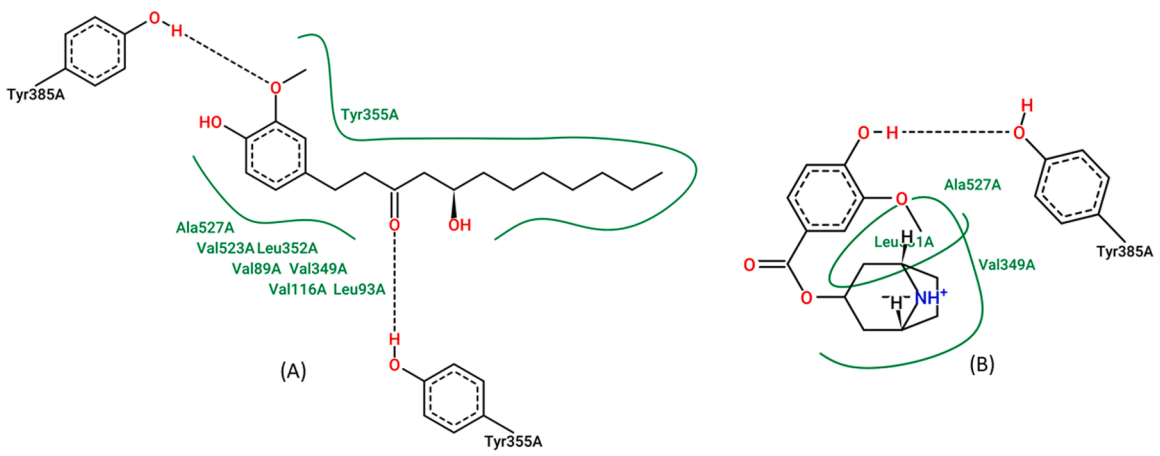


Fig. 4. Interactions obtained from the docked poses of (A) (8)-gingerol and (B) phyllabine with COX-2 enzyme.

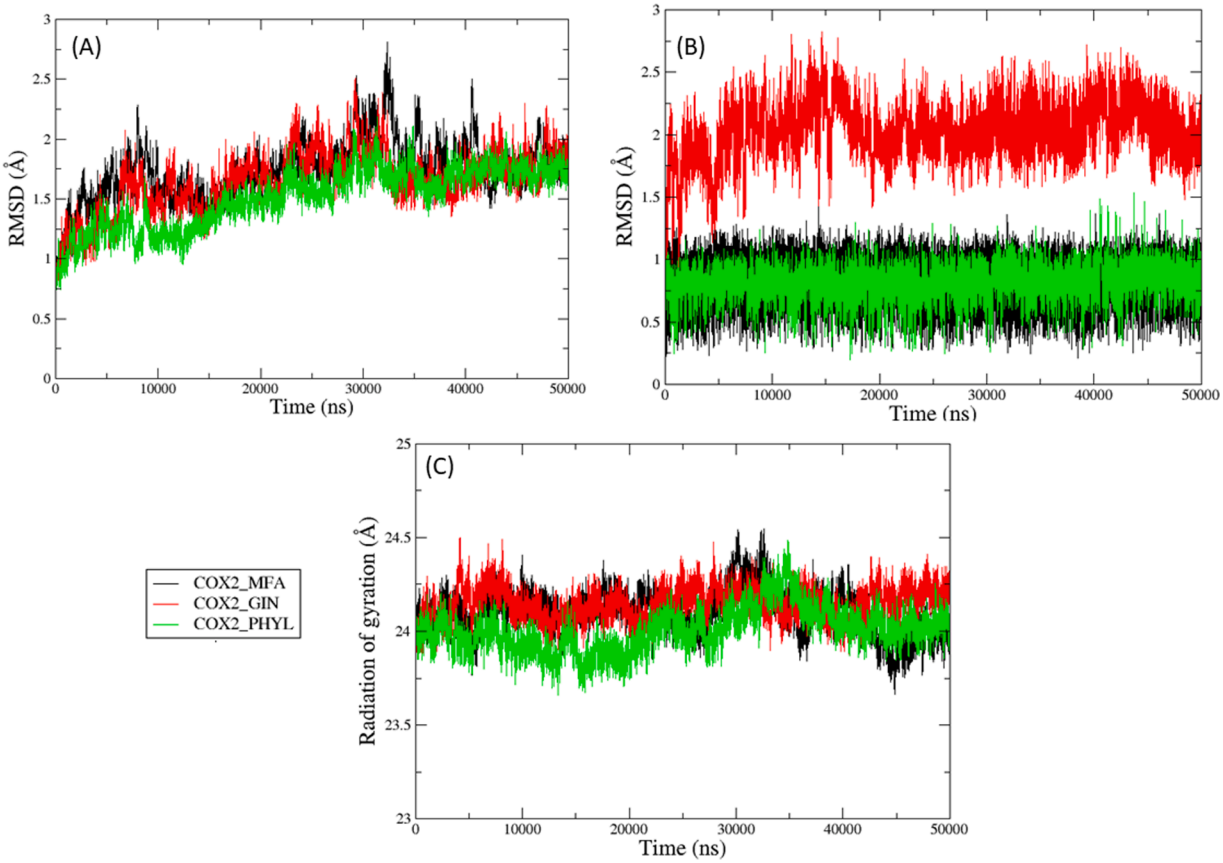


Fig. 5. RMSD plots of (A) protein complexes and (B) bound/docked ligands as well as (C) radiation of gyration of protein complexes in 50ns MD simulations.

Table 11
Calculated binding free energies [$\Delta G_{bind}(T)$] of the compounds in complex with COX-2 enzyme.

Compounds	ΔE_{vdW}	ΔE_{elec}	ΔG_{gas}	ΔG_{polar}	$\Delta G_{nonpolar}$	$\Delta G_{solvation}$	$-T\Delta S$	$\Delta G_{bind}(T)$
(8)-Gingerol	-57	-8.76	-65.76	19.63	-7.62	12.01	30.54	-23.21
Phyllalbine	-43.32	-1.11	-44.43	124.54	-4.87	4.89	18.22	-20.10
Mefenamic acid	-39.66	-5.5	-45.16	10.16	-5.04	5.11	10.78	-29.27

The energy components are in kilocalories per mole (kCal/mol)

Analgesics that involve action on central nervous system are known to increase the threshold required for the activation of nociceptors (Ishola et al., 2011); hence, agents increasing latency of animals to thermal stimulus suggest involvement of central mechanism (Choi and Hwang, 2003; Owoyele et al., 2009). Hot plate test and tail immersion test are sensitive acute pain tests for assessment of analgesic reactions

from central or spinal origin. The results showed that TG did not show any significant ($p>0.05$) change in reaction time in both models whereas pentazocin, being centrally acting drug, showed analgesic effect by increasing the reaction time as compared to vehicle treated rats. This suggests that *trayodashang guggulu* has no influence on centrally mediated perception of pain.

Formalin-induced pain is a model of persistent pain and bears high resemblance to human pain situation (Coelho et al., 2005). Formalin produces a distinctive biphasic reaction initiated by non-inflammatory neurogenic pain (early phase) due to direct chemical stimulation of the nociceptors, particularly C-fibres (Coelho et al., 2005) followed by inflammatory pain (Hunskaar and Hole, 1997) due to the release of several mediators of inflammation like prostaglandins and serotonin (Imam et al., 2012). So, this model can differentiate the mechanisms involved in analgesic action of any agent (Tjolsen et al., 1992).Formalin causes activation of 5-HT1, 5-HT2 and 5-HT4 receptors which are involved in edema formation (Doak and Sawynok, 1997). Formalin also induces cyclooxygenase-2 without affecting cyclooxygenase-1 to develop edema (Yamamoto and Nozaki-Taguchi, 2002). In the present investigation, the formalin administration showed both phase 1 and phase 2 flinching as well as paw licking response in rats. Results showed that TG significantly decreased pain in both early and late phase indicating that TG has antinocipetive effect on both neurogenic and inflammatory types of pain caused by formalin. The standard drug, indomethacin could inhibit only phase 2 flinching and paw licking suggesting its influence on inflammatory pain only (Hunskaar and Hole, 1987). The inhibition of inflammatory pain by TG also indicates its influence on inflammatory mediators like prostaglandin released in the late phase. Besides amelioration of inflammatory pain; it is, however, less understood that TG inhibited neurogenic pain in early phase of nociception as opposed to its negative effect on acute pain in thermal models of nociception. This suggests that TG has weak analgesic effect on acute pain. It is also possible that TG may have direct influence on stimulation of peripheral nociceptors by formalin and may be affecting different transduction molecules viz. substance P and bradykinins other than those involved in central nociception. Earlier experimental studies indicated that formalin predominantly evokes activity in unmyelinated slow conducting C fibres and not in myelinated fast conducting A δ fibres (Heapy et al., 1987) which are predominantly sensitive to thermal and/or mechanical stimulus (Lewin and Moshourab, 2004; Cain et al., 2001). This further suggests that TG might be affecting early phase nociception of formalin through its action on C afferents. The above antinociceptive effect of TG in formalin-induced pain substantiates its therapeutic use in persistent and recurrent tonic pain conditions like arthritis, sciatica, ligament injury, etc.

Formalin causes peripheral tissue inflammation. In formalin-induced paw edema model, the TG and indomethacin treatment significantly prevented the increase in paw volume after formalin administration which indicates anti-inflammatory property of the formulation. Carrageenan-induced inflammation is also a biphasic model where the early phase is characterized by the release of inflammatory mediators likes histamine and serotonin while the late phase is marked by increased production of prostaglandins (Nantel et al., 1999). In the late phase, the excess production of prostaglandins causes vascular permeability and edema which are blocked by NSAIDs (Handy and Moore, 1998). In the present study, the TG and indomethacin treatment significantly prevented the increase in paw volume at 3^{rd} and 5^{th} h without any effect at 1^{st} h which supported our contemplation of anti-inflammatory nature of TG. The inhibitory action of TG on the prostaglandin mediated late phase of inflammation suggests its plausible influence on prostaglandin pathway of inflammation. However, the exact mechanism of anti-inflammatory action of TG is difficult to elucidate as it contains multiple ingredients which may influence different targets and elicits multitargeted action. Our previous *in-vitro* study revealed that *trayodashang guggulu* exhibited membrane stabilizing action and inhibition of protein denaturation as well as inhibition of inflammatory enzymes i.e. protease and lipoxygenase (Dadoria et al., 2020) which might be contributing to the probable mechanism of anti-inflammatory effect of TG.

Phytochemical studies revealed the presence of fair amount of total phenolics and flavonoids in *Trayodashang guggulu*. Many flavonoids and phenolic compounds were reported to have anti-inflammatory activities (Luo et al., 2002; Okoli and Akah, 2004). The plants constituents of TG have anti-inflammatory properties and containphenolics and phytosteroids as major constituents Jeet and Thakur, 2012; Battu and Kumar, 2010; Chawla et al, 1991). Thus, the presence of flavonoid and phenolic compounds might be contributing to anti-inflammatory property. Previous studies reported that various other phytochemicals were also isolated from constituent plants of the TG formulation like ψPS-taraxasterol acetate (*Pluchea lanceolata*) (Shrivastava et al., 1990), sesquiterpens, diterpens and diterpenic acid from *Commiphora myrrha* (Su et al., 2011; Jain and Gupta, 2006), withaferin from W*ithania somniphera*(Khare, 2007), and 6 shagol (Levy et al., 2006) and gingerol (Young et al., 2005) from *Zingiber officinale* exhibited anti-inflammatory activities. Hence, the anti-inflammatory action of TG may be attributed to the presence of these anti-inflammatory plant ingredients. In the present study the LC-MS analysis revealed the presence of 17 phytoconstituents in TG i.e. Myricanol5-laminaribioside, Quercetin 3-sambubioside, Rutin, Theaflavate A, Gitoxin, Proanthocyanidin A2, Kaempferol 3-(2′',3′'-diacetyl-4′'-p-coumaroylrhamnoside), Schidigerasaponin B1, Phyllalbine, Bufotenine O-glucoside, Rosmarinine, (8)-Gingerol, Gingerglycolipid C, Linalool oxide D 3-[apiosyl-(1->6)--glucoside], Cinncassiol D4, ent-Epicatechin-(4alpha-->8)-ent-epicatechin 3-gallate. Further, the predictions from *in silico* studies help to understand the interactions between these phytochemicals and COX-2 from druggability scores, similarity search analyses, molecular docking and MD simulations. The current analysis indicate that at least some of these phytochemicals may have activity against these enzymes and synergistic effects may be responsible for the obtained anti-inflammatory properties of their mixtures obtained from the constituent plants. Based on the current findings, further experimental research needs to be instigatedto identify the exact inflammatory pathway influenced by *trayodashang guggulu* for ameliorating inflammation and associated pain.

Conclusion

In conclusion, *trayodashang guggulu* inhibited the inflammatory pain as well as showed anti-inflammatory activity in rats. The effect may be attributed to the presence of anti-inflammatory phytoconstituents through the inhibition of anti-inflammatory enzymes like COX-2. The study strengthens the therapeutic claim of the formulation as anti-inflammatory drug in Ayurvedic system of medicine and substantiates its use against painful inflammatory conditions like sciatica, arthritis, gout, etc.

Conflict of Interest

Authors declare no conflict of interest.

Conflict of interest declared

None

Author's contribution

Manish M Wanjari and Pushpendra Kannojia contributed in the concept and design of the research work. Shivani Gupta, Yadu Nandan Dey and Deepti Sharma executed experimental studies. Amit Kumar Halder executed the in silico studies. Sharad Pawar and Shridhar Chaugule carried out LCMS analysis. Yadu Nandan Dey and Manish M Wanjari executed statistical analysis. Yadu Nandan Dey, Shivani Gupta

and Manish M Wanjari prepared the manuscript. Atul Kaushik, Sudesh N Gaidhani and Shailendra Gurav contributed in reviewing and revising the manuscript. All authors have read and approved the manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Supplementary materials

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