ORIGINAL ARTICLE

Effect of Shankhpushpi on Alcohol Addiction in Mice

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ABSTRACT

Alcohol addiction is a worldwide problem. It has mainly two components: dependence and withdrawal. Characteristic properties of most anti-addictive compounds include anti-anxiety, anticonvulsant, antidepressant, and nootropic actions. Shankhpushpi (*Convolvulus pluricaulis*. Convolvulaceae), known ethnopharmacologically as brain tonic, possess all the properties mentioned above. Here, we screen shankhpushpi for possible anti-addictive potential. Effect of shankhpushpi churna was measured on ethanol withdrawal anxiety using elevated plus maze. The role of shankhpushpi on chronic ethanol consumption (21 days) was measured using two bottle choice protocol of voluntary drinking. We also measured the effect of the above herb on cortico-hippocampal GABA levels. Shankhpushpi was found to reduce alcohol withdrawal anxiety in a dose-dependent manner. The herb also decreased ethanol intake and increased water intake significantly ($P < 0.001$) after 4 days of administration. Both these effects were blocked ($P < 0.001$) by GABA$_A$ antagonist suggesting the role of GABA$_A$ receptor. Chronic administration of shankhpushpi also significantly ($P < 0.01$) increased cortico-hippocampal GABA levels in mice. Shankhpushpi reduced both alcohol dependence and withdrawal in a GABA$_A$-dependent manner, thus showing anti-addictive potential.

Key words: Alcohol, addiction, *Convolvulus pluricaulis*, shankhpushpi, mice

SUMMARY

- Shankhpushpi prevented ethanol withdrawal anxiety and alcohol addiction in a GABA$_A$-dependent manner.

SHANKHPUSHPI
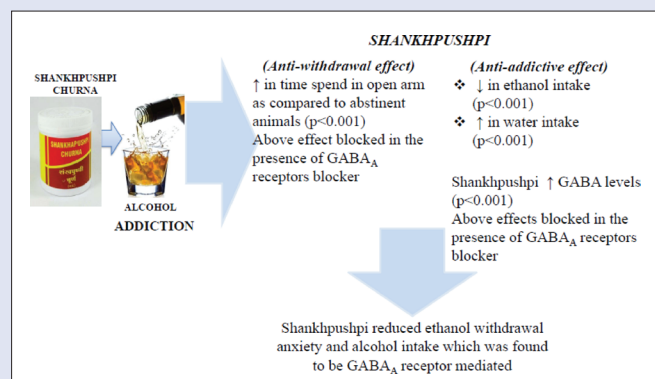
Abbreviations used: GABA: Gamma-Aminobutyric Acid, HIV: Human Immunodeficiency Virus, CNS: Central Nervous System

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INTRODUCTION

Alcohol addiction is a worldwide problem. Alcohol consumption accounts for more than 3.3 and is the fifth leading risk factor for premature death and disability. Alcohol contributes various diseases and injury-related health conditions, most notably alcohol dependence, liver cirrhosis, cancers, HIV, and injuries, primarily road traffic accidents.[1,2] Alcohol dependence is a complex and dynamic process involving various neurobiologic and environmental factors.[3] Individual's propensity to alcohol consumption is primarily a balance between alcohol's rewarding effects and its withdrawal consequences. One factor contributing to relapse is withdrawal related anxiety, which likely reflects adaptive changes in the brain in response to continued alcohol exposure. Association of these pleasant/unpleasant feelings with environmental clues may influence alcohol intake.[4] Alcohol withdrawal symptoms include irritability, agitation, anxiety, sleep disturbances, and reduced pain threshold both in human and animals.[5] Disulfiram, naltrexone, and acamprosate are US Food and Drug Administration (US-FDA) approved medications for treatment of alcohol dependence. However, they have various side effects including palpitation, flushing, nausea, vomiting, headache, anxiety, sedation, and transient diarrhea, which affect the quality of life of the addicted individual under anti-addictive therapy.

Herbal medicines can be used for the development of new therapeutically active compounds with higher potency and lower toxicity. Most modern drugs have a natural origin and play a major role in drug development.[6] Shankhpushpi (*Convolvulus pluricaulis Choisy*) is a perennial herb referred to as morning glory. The plant is used locally in Indian and Chinese system of medicine to treat various diseases. Its different pharmacologic actions include cough suppressant, antihypertensive, antiulcer, hypolipidemic, and against various neurologic disorders. It may be used as a stress reliever, a mood stabilizer, and memory enhancer. It may also reduce peripheral nerve hyperexcitability, as well as used as an adjuvant to anticonvulsant therapy.[7,8] Its primary chemical constituent

includes alkaloids like shankhpushpine, convolamine, convoline, convolidine, convolvine, confoline, convosine, and so on, found in different species from this family. The fresh plant contains volatile oils, fatty acids, fatty alcohols, and hydrocarbons like myristic acid, palmitic acid, linoleic acid, and straight-chain hydrocarbons like hextriacontane. The flavonoid kampferol and steroids phytosterol, β-sitosterol have also been extracted.[7,9] Many of these active constituents isolated from aerial parts of *C. pluricaulisy* like shankhpushpine, scopoletin, kaempferol phytosterol, and β-sitosterol have been shown to act as GABA$_A$ agonists, which could be attributed to the anxiolytic and CNS-depressant actions of shankhpushpi.[10]

Here, we report that acute administration of shankhpushpi may prevent alcohol withdrawal anxiety, whereas its chronic administration may reduce alcohol consumption in mice. Chronic administration of shankhpushpi extract led to change in cortico-hippocampal GABA levels, whereas GABA$_A$ blocker completely reversed its anti-addictive properties. Thus, above results suggest that *C. pluricaulis* may prevent alcohol addiction in a GABA$_A$-dependent manner.

MATERIALS AND METHODS

Animals

Swiss albino mice (20–30 g) were used in this study. Animals were issued from the Institutional Animal House (Reg. No. 621/02/AC/CPCSEA) of Birla Institute of Technology, Mesra. All animals were kept in polyacrylic cages and maintained under standard conditions (room temperature 24–27°C and humidity 60–65% with 12:12 light:dark cycles). The food was provided in the form of dry pellets and water *ad libitum*. The animals were allowed to get acclimatized to the laboratory conditions for 7 days before the commencement of the experiment. All experiments involving animals complied with the ethical standards of animal handling and approved by the Institutional Animal Ethics Committee (BIT/PH/IAEC/28/2013).

Estimation of blood alcohol levels

Blood was collected by retro-orbital bleeding with animals in light ether anesthesia after 20 min of ethanol administration. Ethanol levels were measured using ultraviolet (UV) assay kit for alcohol estimation based on manufacturer's protocol (Thermo Fisher Scientific (India) Pvt. Ltd).

Development of conditioned place preference model

Apparatus

The conditioned place preference (CPP) apparatus contain three compartments. The two end compartments (30.5 cm × 26.5 cm × 37 cm) were connected by a central corridor (12.75 cm × 23 cm × 15.25 cm). The compartment on the left had black walls with a perforated stainless steel floor with round holes on staggered centers. The central corridor was transparent with a smooth plexiglass floor, and the right compartment had white walls with a stainless steel mesh floor.

CPP was performed as described previously,[11,12] with slight modifications. It mainly consists of three phases:

(1) Preconditioning phase: (first and second day) The animals were placed in the middle chamber and allowed to explore both the chambers for 30 min.

(2) Conditioning phase: (3rd - 10th day) Each mouse was treated for eight consecutive sessions with the alternate oral administration of alcohol and saline. On days 3, 5, 7, and 9, the animals were administered ethanol (2 g/kg body weight; i.p. 10% [v/v]) and placed in one compartment for 30 min. Besides, on days 4, 6, 8, and 10, the animals were administered

saline and placed in opposite compartment.

(3) Postconditioning phase: (11th–12th day) Mice were placed in the middle chamber and allowed free access to both chambers for 30 min. Time spent in ethanol and saline-paired chamber was measured.

(4) Treatment protocol: After development of withdrawal (15th day), the following treatment schedule was followed:

Group 1: saline

Group 2: ethanol

Group 3: ethanol + shankhpushpi (Vyas Pharmaceuticals, Haridwar; 100 mg/kg)

Group 4: ethanol + shankhpushpi (200 mg/kg)

Group 5: ethanol + diazepam (1 mg/kg)

Group 6: ethanol + GABA$_A$ antagonist

Group 7: ethanol + GABA$_B$ agonist

Group 8: ethanol + shankhpushpi + GABA$_A$ antagonist

Group 9: ethanol + shankhpushpi + GABA$_B$ agonist

The behavioral tests were performed 60 min after oral drug administration and 30 min after intraperitoneal administration.

Behavioral studies to measure alcohol withdrawal anxiety

Elevated plus maze

The model has been validated pharmacologically and currently considered the "gold standard" test of anxiety-related behavior. Elevated plus maze (EPM) was performed as described by Kokare *et al*.[13] In summary, after drug treatment, individual mice were placed at the center of the maze, head facing an open arm. During the 5 min test period, the number of entries and time spent on the open arm were recorded automatically (Medicraft Electromedical, Lucknow, India).

Chronic-treatment study to measure alcohol intake

Two-bottle choice ethanol drinking

We used the standard two-bottle choice protocol, which is a widely used animal model to capture aspects of voluntary alcohol consumption in humans.[14] Following 7 days of acclimatization, animals were subjected to an ethanol drinking acquisition regimen. The animals remained in their home cages at all times throughout the study but had their water bottles removed during a 4 h and ethanol presentation period. During this time, animals were exposed to a free choice between ethanol (15% v/v) and water for 20 days but with no drug pretreatment.

After 20 days of ethanol administration, animals were divided into different groups for 10 days of treatment. Each day, the bottles were weighed before and after 4 h of limited access period and the differences were used to calculate the water and ethanol intake. The mean intake was expressed as g/kg body weight/day of water and g/ kg body weight/day of ethanol intake. All animals were given unrestricted food access. Every 2 days, the bottles were switched to eliminate place preference.[15] After 20 days of pretreatment with ethanol (15% v/v), the animals were divided into different treatment groups (*n* = 7 per group) as follows:

Group 1: (control) received saline 30 days

Group 2: received free choice ethanol (15% v/v)/water 30 days

Group 3: received free choice ethanol (15% v/v)/water and shankhpushpi (200 mg/kg) 21st–30th day

Group 4: received free choice ethanol (15% v/v)/water and diazepam 21st–30th day.

Group 5: received free choice ethanol (15% v/v)/water, GABA$_A$ antagonist and shankhpushpi 21st–30th day.

After the above experimental protocol of 30 days, five animals per group were sacrificed under ether anesthesia by cervical dislocation for biochemical estimation.

Estimation of gamma amino butyric acid levels from brain tissue

Brain tissue was homogenized in 5 mL of 0.01 M HCl. In this homogenate, 8 mL of ice cold ethanol was added and kept for 1 h at 0°C. The contents were centrifuged for 10 min at 16,000 rpm and supernatant was collected in a petri dish. The precipitate was washed three times with 5 mL of 75% ethanol. The washes were combined with supernatant and evaporated to dryness. To the dry mass, 1 mL water and 2 mL chloroform were added and centrifuged at 2000 rpm. Upper phase containing GABA was separated, and 10 μL of it was applied as spot on Whatman filter paper.

The mobile phase consisted of *n*-butanol, acetic acid, and water in 4:1:5 ratios. The chamber was saturated for half an hour with mobile phase. The paper chromatogram was developed with ascending technique. The paper was dried in a hot air oven and then sprayed with 0.5% ninhydrin solution in 95% ethanol. The paper was dried. Blue spot developed on paper, which was cut and heated with 2 mL ninhydrin solution on a water bath at 60–65°C. Water was added to the solution and kept for 1 h and supernatant was used. Absorbance was measured at 570 nm on a UV-visible spectrophotometer.

RESULTS

CPP

In CPP, the animal's choice to spend more time in either environment provides a direct measure of the conditioned reinforcing effect of a drug. Animals were found to prefer the ethanol-paired chamber over the saline-paired chamber. In our study on day 11, the ethanol-treated animals spent significantly more time in the ethanol-paired chamber as compared to the saline-paired chamber ($P < 0.001$). Animals spent about 66% of total time in ethanol-paired chamber versus the saline-paired. For the control group (saline-treated), the time spent in
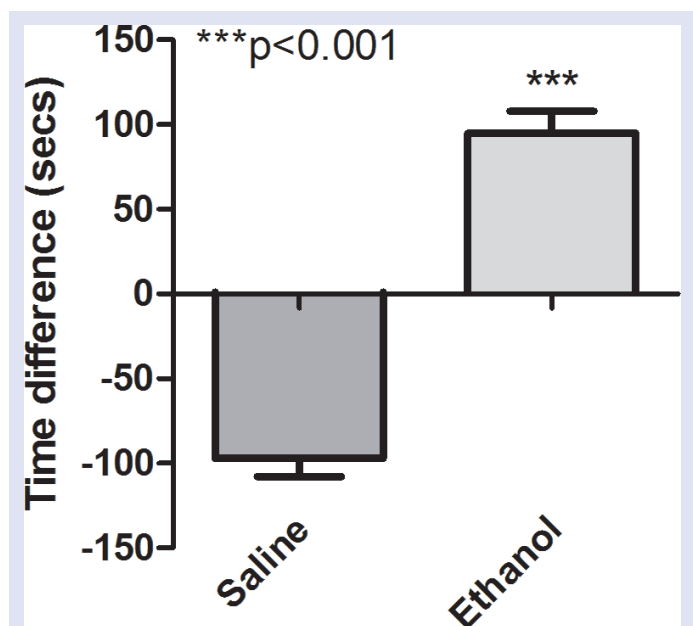
both the chambers were comparable [Figure 1]. Similar results were observed on day 12 (data not shown). The CPP results suggested that the animals got addicted to alcohol. Ethanol levels in the blood samples were found to be 47 mg/dL ($n = 6$).

Effect of shankhpushpi on withdrawal anxiety

In the present study, according to the CPP model described by Thanos *et al.*,[11] with minor modifications, acute ethanol withdrawal anxiety was developed and measured using elevated plus maze test. Five days of abstinence followed 10 days of conditioning phase in which alternate dose of ethanol and saline was given for 10 days. After 5 days of abstinence from ethanol, animals showed a significant decrease in time spent ($P < 0.01$) in the open arm of the elevated plus maze as compared with the control, suggesting withdrawal anxiety. Shankhpushpi (100 and 200 mg/kg) administration led to a dose-dependent reversal of withdrawal anxiety as evidenced by significant increase in time spent in the open arm ($P < 0.01$).

Both 200 mg/kg shankhpushpi and diazepam showed comparable anxiolytic potential against ethanol withdrawal anxiety. However, pretreatment with GABA$_A$ antagonist prevented shankhpushpi-mediated reversal of withdrawal anxiety ($P < 0.001$) [Figure 2]. Treatment with GABA$_B$ agonist and shankhpushpi did not show any significant change in withdrawal anxiety compared with shankhpushpi-treated animals (data not shown). The above results suggest that shankhpushpi may
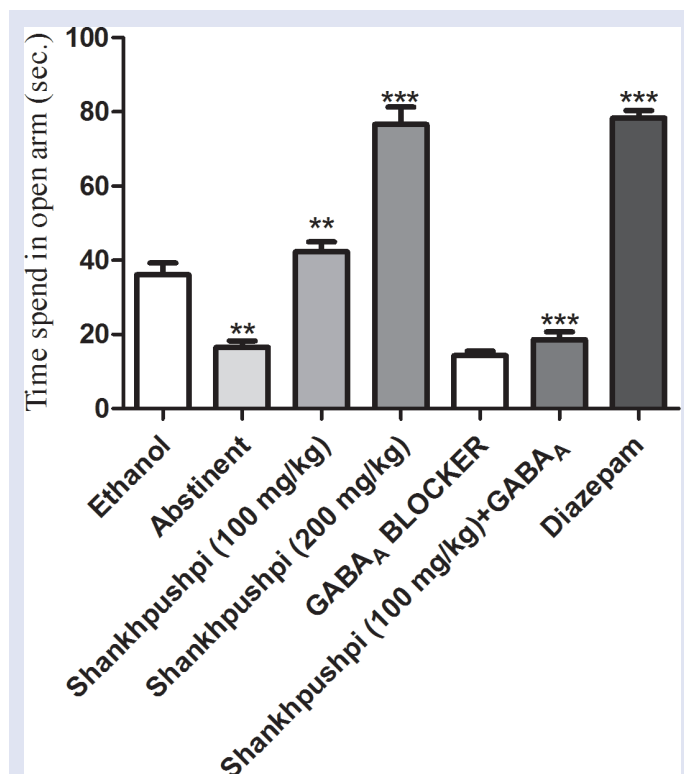
Figure 2: Effect of shankhpushpi on ethanol withdrawal anxiety using elevated plus maze. Ethanol abstinence significantly decreased ($P < 0.001$) time spend in open arm compared with ethanol-treated animals. Shankhpushpi treatment to abstinent animals significantly (100 mg/kg, $P < 0.01$ and 200 mg/kg, $P < 0.001$) increased time spend in open arm compared with abstinent animals. Diazepam also significantly increased time spend in open arm over abstinent and ethanol-treated groups ($P < 0.001$). Animals treated with shankhpushpi in the presence of GABA$_A$ blocker spent significantly less time in the open arm ($P < 0.001$) compared with abstinent animals.

Figure 1: CPP on ethanol administration. Time spent in alcohol and saline administration chamber on day 11 by control and ethanol-treated group. Ethanol-treated animals showed significant increase in ($P < 0.001$) time spent in ethanol-paired alcohol compared with saline-paired chamber. Values represent mean ± standard error of the mean $n = 7$
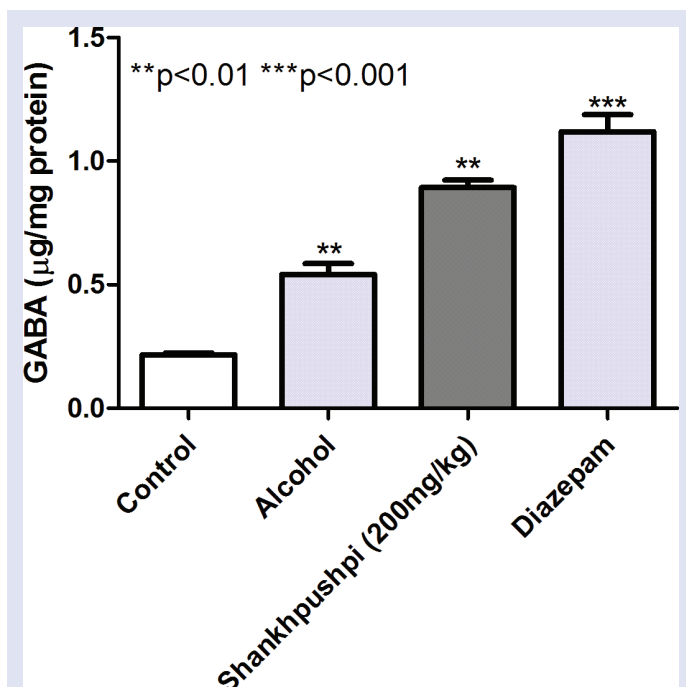
Figure 3: Effect of shankhpushpi on cortico-hippocampal GABA levels. Shankhpushpi (200 mg/kg) treatment (10 days) led to significant increase ($P < 0.01$) in cortico-hippocampal GABA levels compared with untreated animals on ethanol (30 days). Diazepam treatment also showed a significant increase ($P < 0.001$) increase in cortico-hippocampal gamma amino butyric acid levels. Values represent mean ± standard error of the mean $n = 5$

reverse ethanol withdrawal anxiety in a GABA$_A$-dependent manner.

Effect of shankhpushpi on cortico-hippocampal GABA levels

After 30 days of alcohol treatment, animals showed a significant increase ($P < 0.01$, $n = 5$) in cortico-hippocampal GABA as compared with control group. Shankhpushpi (200 mg/kg) treatment (10 days) showed a significant ($P < 0.01$, $n = 5$) increase in GABA as compared with alcohol-consuming animals. This significance further increased ($P < 0.001$, $n = 5$) in diazepam-treated group as compared with alcohol group [Figure 3].

Effect of shankhpushpi on alcohol consumption

Shankhpushpi-treated animals showed a significant ($P < 0.001$, $n = 7$) decrease in ethanol and water intake as compared with the control group after day 24 or 4 days postshankhpushpi therapy. This was comparable with diazepam-treated animals, who also showed a significant decrease ($P < 0.001$, $n = 7$) in ethanol intake. However, animals' administration with GABA$_A$ blocker followed by shankhpushpi failed to show a decrease in ethanol and increase in water intake till day 30. The above results suggest that shankhpushpi prevented chronic ethanol intake, which may be mediated by GABA$_A$ receptors [Figure 4].

DISCUSSION

In the present work, we study the effect of shankhpushpi in both acute alcohol withdrawal and chronic alcoholism. Addiction model used in the alcohol-withdrawal study was CPP. The CPP results suggest that when given a choice after administration of alcohol for 11 days, the animals preferred the ethanol-paired chamber to that of saline-paired chamber, thus confirming the development of addiction. Ethanol consumption followed by withdrawal results in the development of abstinence syndrome.[14–18] Common and prominent feature of alcohol withdrawal is anxiety, which is also considered to be the most important negative motivation to revert to alcohol use.[19] The above sign of ethanol withdrawal (EW) have been attributed to upregulation of NMDA receptors[20] and downregulation of GABA$_A$ receptors.[21] Therefore, a drug that either facilitates the action of GABA or decreases glutamate activity may be effective in EW-induced anxiety behavior. The elevated plus maze is the most commonly employed tests for assessing anxiety-like behavior after alcohol withdrawal, which is a measure of psychologic dependency.[22] Typical anxiolytic drugs increase the proportion of entries, time spent, and rearing in the open arms. The anxiolytic potential of shankhpushpi churna and diazepam were studied on acute ethanol withdrawal anxiety using elevated plus maze. Abstinence to ethanol led to precipitation of withdrawal anxiety as revealed by the significant reduction in time spent in open arm of EPM. Shankhpushpi churna and diazepam increased the time spent in open arm, thus reducing ethanol withdrawal anxiety, one of the primary reasons for alcohol addiction.

Benzodiazepines are a positive allosteric modulator of GABA$_A$. They act by potentiating the effect of GABA on its receptor.[23] It has been suggested that downregulation of GABA$_A$ receptor and/or decrease in the GABAergic transmission may be responsible for ethanol withdrawal. Diazepam acts by potentiating the effect of GABA at the receptor site and shows its anxiolytic property. We also found a significant increase in GABA levels upon administration of shankhpushpi churna and diazepam. Treating animals with GABA$_A$ antagonist successfully reversed the anxiolytic property of shankhpushpi, thus pointing toward the role of GABA$_A$ receptor in shankhpushpi-mediated reduction in withdrawal anxiety.

Next, we determined the effect of shankhpushpi on alcohol consumption in mice. The two-bottle choice protocol is a widely used model that captures aspects of voluntary alcohol consumption in humans.[24] After 30 days of chronic ethanol intake, animals showed increased cortico-hippocampal levels of GABA as we reported previously.[25] Decrease in GABAergic function after chronic administration of alcohol in experimental animals has been widely attributed to decrease in GABA$_A$ receptor expression and function.[26] Shankhpushpi treatment significantly increased GABA levels in the cortico-hippocampal lysates over ethanol-treated animals. Diazepam, a standard anxiolytic drug also showed increased GABA levels. Others have also reported the role of GABA in anxiolytic property of aerial parts of *C. pluricaulis*.[27] Alcohol is an indirect GABA agonist. Plasma and CSF level of GABA have been found to remain high after initial withdrawal than after longer periods of abstinence.[28] Evidence showed a decrease in GABAergic function after chronic administration of alcohol in experimental animals, which has been attributed to decrease in the GABA$_A$ receptors or changes in the composition of the receptor.[26]

Ethanol intake by mice was found to increase till the 20th day. After day 24, there was a significant decrease in ethanol intake and increase in water intake for shankhpushpi and diazepam, treated animal which was completely blocked upon administration of GABA$_A$ blocker The above results suggest that shankhpushpi may prevent ethanol intake in a GABA$_A$-dependent manner. Alcohol's action on GABA$_A$ receptors strongly depends on its subunit composition. GABA$_A$ receptors are composed of α, β, γ, and δ subunits forming a pentameric ligand-gated ion channel receptor. While most subunit compositions of GABA$_A$ receptors display responses to alcohol only at high concentrations (460 mM), it has been found that lower concentrations (1—3 mM) of alcohol may only alter the activity the δ subunit.[29] Pharmacologic manipulation of GABA$_A$ receptors has been studied in mice. Negative allosteric modulators of
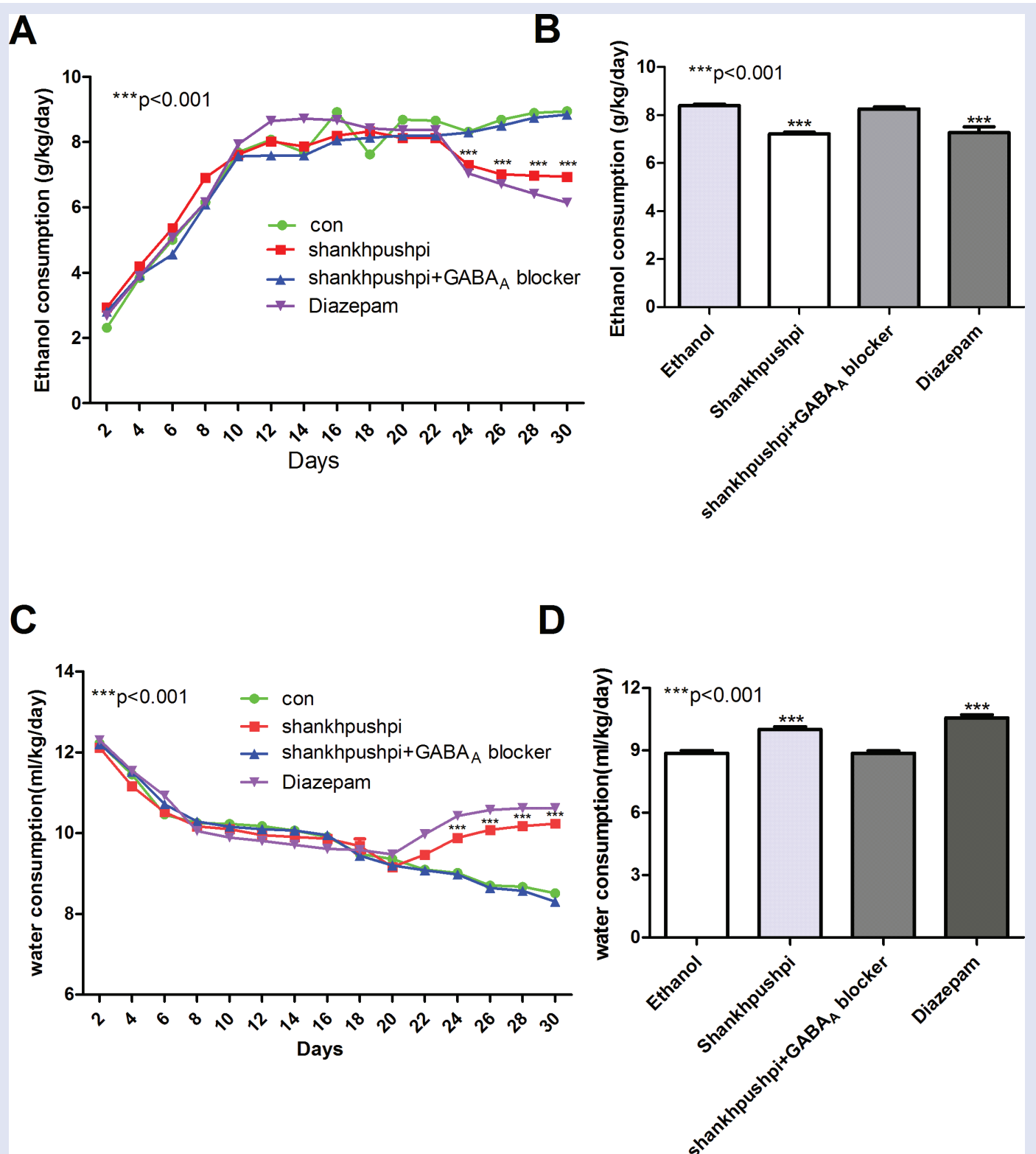
Figure 4: Effect of shankhpushpi on ethanol intake. (a) Changes in the ethanol intake before and after treatment. Shankhpushpi- and diazepam-treated animals showed a significant decrease in ethanol intake compared with untreated ethanol consuming animals from day 24 ($P < 0.001$, $n = 7$) or after 4 days of treatment. In presence of GABA$_A$ blocker shankhpushpi showed little decrease in ($P > 0.5$) ethanol intake compared with ethanol-consuming animals till day 30. (b) Changes in ethanol intake on day 24 of the 30 days study. All treatment groups other than GABA$_A$ blocker + shankhpushpi group showed significant ($P < 0.001$) decrease in alcohol intake. (c) Change in the water intake before and after treatment. Shankhpushpi (200 mg/kg) and diazepam-treated animals showed a significant increase in water intake compared with untreated ethanol-consuming animals from day 24 ($P < 0.001$, $n = 7$) or after 4 days of treatment. In presence of GABA$_A$ blocker shankhpushpi did not show an increase ($P > 0.5$) in water intake compared with ethanol-consuming animals. (d) Change in alcohol intake on day 24 of the 30 days study. All treatment groups other than GABA$_A$ blocker + shankhpushpi group showed significant ($P < 0.001$) increase in water intake. Values represent mean ± standard error of the mean, $n = 7$

the $GABA_A$ receptor have been shown to reduce alcohol consumption in several alcohol-preferring mice.[21] Various phytoconstituents identified from the aerial parts of *C. pluricaulisy Choisy* may act as $GABA_A$ agonist; this may be attributed to its CNS-depressant actions.[30,31]

CONCLUSION

Hence, in the present study, we report anti-addictive potential of shankhpushpi churna in alcohol addiction in mice. It not only reversed ethanol withdrawal anxiety but also decreased chronic alcohol consumption in these animals in a $GABA_A$ receptor-dependent manner.

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Conflicts of interest

There are no conflicts of interest.

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