Investigation of the Anxiolytic Effects of Xanthohumol, a Component of Humulus lupulus (Hops), in the Male Sprague-Dawley Rat

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The purpose of this study was to investigate the anxiolytic effects of xanthohumol, a component of Humulus lupulus (hops), and its potential interaction with the benzodiazepine binding site on the γ-aminobutyric acid (GABA$_A$) receptor in the male Sprague-Dawley rat. This was a prospective, randomized, between-subjects experimental study. Fifty-five rats were assigned to 1 of 5 groups with 11 rats per group: control (vehicle), xanthohumol, midazolam, midazolam with xanthohumol, and flumazenil with xanthohumol. In this study the elevated plus maze measured the behavioral components of anxiety and motor movements. A 2-tailed multivariate analysis of variance and least significant difference post hoc test was used to determine if a significant difference existed. Our data suggest that xanthohumol does not produce anxiolysis by modulation of the GABA$_A$ receptor; however, there may be a possible interaction between xanthohumol and midazolam, or xanthohumol may influence the modulation of another neurotransmitter site in the central nervous system. Alone, xanthohumol does not show significant modulation of the benzodiazepine receptor. Additional research should investigate if xanthohumol acts as a benzodiazepine GABA$_A$ partial agonist or antagonist or if it modulates another neurotransmitter system in the central nervous system.

Keywords: Anxiolysis, hops, Humulus lupulus, Sprague-Dawley rat, xanthohumol.

Anxiety is a physiologic state characterized by cognitive, somatic, emotional, and behavioral components. These components combine to create the feelings that we typically recognize as anger, also known as fear, apprehension, or worry. Anxiety is often accompanied by physical symptoms such as heart palpitations, nausea, chest pain, shortness of breath, stomachaches, and/or headaches. It is estimated that 40 million adults in the United States have some type of anxiety disorder, resulting in costs of more than $42 billion a year.[1] These anxieties range from mild, such as fear of strange situations (eg, operating room) or public speaking, to severe, in which levels are incapacitating and result in job losses. Most patients presenting to the operating room for surgery experience anxiety.

Factors associated with activation of the stress response place the patient at greater risk of adverse outcomes and may lead the patient to have a more complicated anesthetic plan. Activation of the stress response results in a release of both neurotransmitters and hormones. The sympathetic nervous system also is activated, resulting in an elevated heart rate, respiratory rate, and glycolysis and the release of additional catecholamines such as epinephrine and norepinephrine from the adrenal medulla as well as elevated cortisol levels. Prolonged activation of the sympathetic nervous system can lead to fatigue and a weakened immune system.[2]

Anxiety as frequently experienced in perioperative patients places them at greater risk of adverse outcomes. Thus, reducing anxiety before surgery by the administration of anxiety-reducing drugs (eg, benzodiazepines) is an important component of anesthesia; it is necessary to decrease the patient's nervousness in order to decrease both physiologic and emotional negative effects of the stress response. If untreated, the resulting stress response may cause harm to the patient with an already tenuous status, perhaps resulting in an adverse outcome or resulting in poor wound healing. Conversely, it has been shown that when patients have feelings of emotional well-being and decreased anxiety related to surgery, their satisfaction is increased, and they may have shortened recovery times.[3] Additionally, anxiolysis has been shown to be effective in improving postoperative outcomes such as nausea, vomiting, and pain.[4] Anesthesia providers frequently administer anxiolytic medications, such as midazolam, as a means to reduce anxiety, pain, and catecholamine activation.

Many Americans experience anxiety and attempt self-medication with complementary and alternative medicines. These unregulated herbal remedies may have similar mechanisms of action as drugs manufactured for the treatment of anxiety and other disorders. Common undesirable side effects of drugs developed for anxiolysis include hypotension, sedation, and a high potential for addiction and abuse. Conversely, herbal medications are not commonly associated with abuse or addiction.

Herbal medication use has increased rapidly as alternative treatments have multiplied exponentially in America. Research indicates a steady rise in the use of dietary supplements among persons 20 years of age and older: from 1988 to 1994, an increase of 42.1%; 1999 to 2002, a 52.3% increase; and 2003 to 2006, a 53.7% increase.[5] According to the Dietary Supplement Health and Education Act of 1994, there is no requirement for evidence of efficacy, safety, or quality control standards for supplements, which increases the risk of adverse effects related to herbal agents.[6] In the United States between 1993 and 1998, the Food and Drug Administration documented approximately 2,600 adverse events, including 100 deaths related to herbal medications.[7] Currently, there is no central repository for documenting adverse effects associated with herbal remedy interactions; therefore, the true number of adverse effects may be much higher than reported. In fact, because of this undisclosed use of herbal medications, The Joint Commission has mandated a screening for herbal medications at each healthcare visit.[8] The lack of data demonstrates the need for scientific research concerning herbal medications and the possible adverse effects and interactions with perioperative medicines. Many herbal products can interact with frequently used medications, including anesthesia, and may cause serious unforeseen consequences or complications.[9-11]

Patients present for surgery with the belief that herbal remedies are benign and often fail to report their use to anesthesia providers. Herbal medications have become a common alternative to traditional medicines. In the United States between 1990 and 1997, the use of herbal medicine use increased 380%.[7] This steep rise in herbal medication use may be associated with an increase in morbidity and mortality during the perioperative period as a consequence of interactions with prescribed medications or herbal-induced alterations in pharmacodynamics.[9,11,12] Up to 72% of 1,539 adults surveyed regarding their alternative therapies, including herbal use, do not disclose their treatment to healthcare providers, exacerbating the problem.[12] Although herbals have numerous purported benefits, very little scientific research has been conducted concerning their pharmacologic properties.

Hops, or *Humulus lupulus*, have been used for centuries in Europe to add flavor and preserve beer and as a traditional herbal medicine for its mild sedative effects and treatment of nervousness and insomnia.[13] Hops

contain many compounds and constituents, and these constituents are speculated to have important health benefits such as anticancer, anti-inflammatory, and antibacterial properties.[14,15] Further evidence demonstrates sedative effects and possible use as a sleep aid.[15,16] In fact, hop oil extracted from beer was demonstrated to have $GABA_A$ modulation properties and increased pentobarbital-induced sleep time in mice.[17] A principal flavonoid and constituent of hops is xanthohumol (a lipophilic extract) that has been demonstrated to have important anticancer and proestrogen properties.[18] Xanthohumol has also been shown to possibly have anti-infective properties against microorganisms.[19] In addition, xanthohumol has been demonstrated to influence the binding behavior of $GABA_A$ receptors in hippocampal neurons in the rodent model in vitro.[20]

Although there is evidence that demonstrates hops and xanthohumol as containing sedative and central nervous system modulation properties, there is a lack of scientific investigation pertaining to their possible anxiolytic effects. There are no in vivo data describing the potential anxiolytic effects of xanthohumol as the active component of hops.

The aims of this study were as follows:
1. To determine if xanthohumol has anxiolytic effects in the rat model.
2. To discern whether flumazenil antagonizes the anxiolytic effects of xanthohumol in rats.
3. To determine if xanthohumol has an interaction effect with midazolam.

Material and Methods

Hops contain multiple compounds and substances, making it nearly impossible to obtain and administer a standardized hops compound. Therefore, this study evaluated a standardized xanthohumol extract validated by a reputable provider of herbal extracts (Sigma Chemical Co).

A prospective, experimental design using the elevated plus maze (EPM) in the rodent model was used to investigate the objectives of this study. The EPM is a widely used instrument to measure anxiety in the rodent model and has been validated by Treit et al[21] and Pellow et al[22] based on the previous work by Montgomery.[23] Research on the EPM has supported its use as a standard measurement of anxiety and specifically benzodiazepine-induced anxiolysis in rodents.[24] Rats inherently desire to explore a new environment but simultaneously avoid well-lighted and exposed areas (open arms of EPM). Time spent in the open arm represents decreased anxiety exhibited in the rodent model. Time spent in the enclosed arm represents increased anxiety.[21-23]

Power analysis and calculations were performed to determine the minimum number of animals needed to reach statistical significance. The determination of the effect size was based on the literature review and previ-

ous studies that have investigated various compounds for their anxiolytic effects with the EPM. Fifty-five male Sprague-Dawley rats (Harlan Laboratories) weighing 200 to 250 g were used. This research project studied male rats, as female rats would require at least a doubling of total animals used and increased research efforts and costs. Additionally, females have substantial hormonal variations during their estrus cycle, and this may confound results. Normal variations in monthly estrogen levels could have a significant impact on anxiolysis.

The rats were housed in groups of 3 in polycarbonate "shoebox" cages lined with bedding. They underwent a 7-day adaptation period in a temperature-controlled environment (22°C + 1°C, 60% humidity) with a light-dark cycle, receiving 12 hours of light (6 AM to 6 PM) and 12 hours of darkness (6 PM to 6 AM). They were provided free access to food and water. The animals were handled only for weighing, drug administration, and cleaning of cages and were naïve to the EPM instrument. The use of laboratory rats in this protocol was in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the Institutional Animal Care and Use Committee at the US Army Institute of Surgical Research at Fort Sam Houston, Texas.

Rats were assigned to 1 of 5 treatment groups (11 rats per group) by the use of a random numbers table. Each animal received an intraperitoneal injection of one of the following: (1) vehicle (dimethyl sulfoxide [DMSO], Sigma Chemical Co) as a negative control; (2) xanthohumol (Sigma Chemical Co), 20 mg/kg (based on previous work by Avula et al[25]), dissolved in DMSO; (3) midazolam (Roche), 1.5 mg/kg, as a positive control group comparator; (4) flumazenil, 3 mg/kg (Sigma Chemical Co), dissolved in DMSO, and xanthohumol, 20 mg/kg, dissolved in DMSO, to examine possible modulation of the benzodiazepine binding site on the $GABA_A$ receptor; or (5) midazolam, 1.5 mg/kg, and xanthohumol, 20 mg/kg, dissolved in DMSO, to investigate possible interaction effects.

Studies suggested that an intravenous xanthohumol dose of 20 mg/kg would be appropriate to examine anxiolysis in our study. Various oral doses of xanthohumol have shown no detectable plasma levels.[25] In those studies, rats remained conscious, with the ability to maintain locomotion, which is essential for participation in the EPM. Thus, we determined that 20 mg/kg was the optimal dose of xanthohumol for rats to maintain the ability to ambulate in the EPM.

Flumazenil is a known benzodiazepine receptor antagonist.[26,27] The flumazenil dose of 3 mg/kg was used based on previous rodent research examining anxiety.[28]

All animals received two 1-mL total volume injections intraperitoneally. Depending on the group, the first injection was either flumazenil or DMSO, the vehicle for flumazenil. The second injection was also group dependent according to the group assignments. Therefore, the vehicle group received 2 injections of DMSO; the xanthohumol group: an injection of xanthohumol and an injection of DMSO; midazolam group: an injection of midazolam and an injection of DMSO; flumazenil plus xanthohumol group: an injection of flumazenil and an injection of xanthohumol; and the midazolam plus xanthohumol group: an injection of midazolam and an injection of xanthohumol.

In addition, all experimentation occurred on a timed schedule between the hours of 9 AM and 3 PM over 2 consecutive days to ensure that each treatment group was exposed to similar variability of corticosterone release related to the circadian rhythm of the animals.

After the 30-minute period following the drug administration, each animal was placed in the center of the EPM located in a lighted room. Each rat was oriented on the EPM facing an open arm and behavioral responses to anxiety were evaluated by the EPM for 5 minutes. The EPM was networked with MotorMonitor software (Kinder Scientific) that tracked the number of entries into each type of arm (open vs closed), time spent in the open arms expressed as a percentage of the total time, and fine and basic motor movements. Basic motor movements are the simple count of beam breaks in the EPM. Each time a photobeam is interrupted, the basic movement count is increased. These movements reveal a gross measure of locomotion but do not distinguish what type of activity is being performed. Fine motor movements are a compilation of small animal movements such as grooming and head weaves or bobs. The EPM was cleaned with nonfragrant soap and water and was dried between each animal trial to limit variability. Immediately following the 5-minute test on the EPM, the animals were removed and placed back in their cages.

Results

Data analyses were conducted using a 2-tailed multivariate analysis of variance and least significant difference post hoc test. Analysis of the ratio of open-arm time vs total time spent in the EPM revealed statistically significant increases between the midazolam and control groups ($P = .002$); midazolam and xanthohumol groups ($P = .004$); and the midazolam and flumazenil plus xanthohumol groups ($P = .004$). However, there was no significance found between the midazolam group compared with the midazolam plus xanthohumol group ($P = .127$; Table; Figure 1).

Total number of basic (gross) and fine motor movements tracked during time in the EPM were analyzed. Analysis showed a significant decrease in basic motor movement of rats in the midazolam group compared with the control group ($P = .004$); the xanthohumol group ($P < .001$); the flumazenil plus xanthohumol group ($P < .001$); and the midazolam plus xanthohumol group ($P = .004$), as shown in the Table and Figures 2 and 3. Similarly, a

Measure	Group				
	Control	Xanthohumol	Midazolam	Flumazenil + xanthohumol	Midazolam + xanthohumol
Ratio of open-arm time/total maze time (s)	5.9 ± 1.5	8.1 ± 2.5	30.6 ± 10.7[b]	8.1 ± 19.9	19 ± 3.3
Basic movements	613 ± 64	795 ± 63	279 ± 64[b]	700 ± 75	615 ± 113
Fine movements	429 ± 41	548 ± 44	192 ± 43[b]	491 ± 52	417 ± 74
Post hoc analysis					
Ratio of open-arm time/total maze time (s)	Midazolam vs control: $P = .002$ Midazolam vs xanthohumol: $P = .004$		Midazolam vs flumazenil + xanthohumol: $P = .004$		
Basic movements	Midazolam vs control: $P = .004$ Midazolam vs xanthohumol: $P < .001$		Midazolam vs flumazenil + xanthohumol: $P < .001$ Midazolam vs midazolam + xanthohumol: $P = .004$		
Fine movements	Midazolam vs control: $P = .002$ Midazolam vs xanthohumol: $P < .001$		Midazolam vs flumazenil + xanthohumol: $P < .001$ Midazolam vs midazolam + xanthohumol: $P = .004$		

Table. Ratio of Open-Arm Time to Total Maze Time and Number of Motor Movements on Elevated Plus Maze per Group[a]
[a] Data are presented as mean ± standard error of the mean.
[b] Statistically significant difference of $P < .05$ on 2-tailed multivariate analysis of variance.

significant decrease in fine motor movement of rats was found in the midazolam group compared with the control group ($P = .002$); the xanthohumol group ($P < .001$); the flumazenil plus xanthohumol group ($P < .001$); and the midazolam plus xanthohumol group ($P = .004$).

Discussion

Although hop is a popular herbal supplement,[14,15] it is suspected that its xanthohumol component exerts central nervous system effects such as sedation and calming. This study examined the purported anxiolytic properties of xanthohumol and its potential interaction with the $GABA_A$ receptor site. The purpose of this study was to evaluate possible central nervous system effects of xanthohumol, a constituent of hops, and potential interactions with midazolam. These findings are not generalizable to patients who are presenting for anesthesia after ingesting beer.

The behavioral measurements comparing the ratio of open-arm time to total time spent in the EPM suggests that xanthohumol alone does not produce anxiolysis. These findings answer the first aim of this study: to determine if xanthohumol has anxiolytic effects in the rat model. Comparing the ratio of open-arm time to closed arm time, there was a significant difference between the midazolam group and all the other groups except the midazolam plus xanthohumol group. This suggests there may be a possible interaction between xanthohumol and midazolam (third research aim), or xanthohumol may influence the modulation of another neurotransmitter site in the central nervous system. Xanthohumol alone, does not show significant modulation of the benzodiazepine receptor; therefore, these data do not support
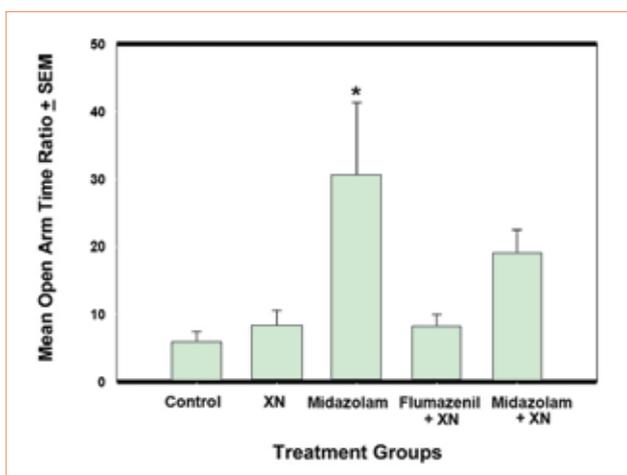
Figure 1. Ratio Open-Arm Time to Total Time on Elevated Plus Maze[a]
Abbreviations: SEM, standard error of the mean; XN, xanthohumol.
[a] Each group was composed of 11 rodents. Drugs were injected 30 minutes before testing on the elevated plus maze.
* $P < .05$.

the hypothesis that xanthohumol modulates the $GABA_A$ receptor resulting in anxiolysis. As xanthohumol did not demonstrate anxiolytic effects, the second aim, which was dependent on the first aim, was retracted. It became impossible to discern whether flumazenil antagonizes the anxiolytic effects of xanthohumol, when no anxiolytic effects were found.

In regard to both basic and fine motor movements, there was a significant difference between the midazolam group and all the other groups. This also suggests that there may be a possible interaction between
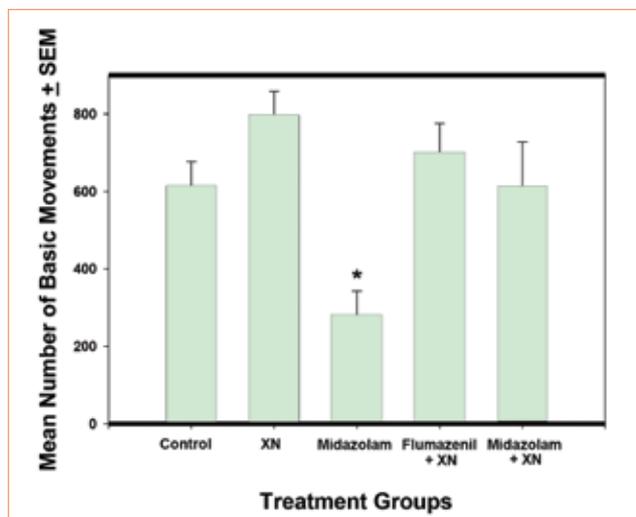
Figure 2. Basic Movements of Rats 30 Minutes After Treatment

Abbreviations: SEM, standard error of the mean; XN, xanthohumol.
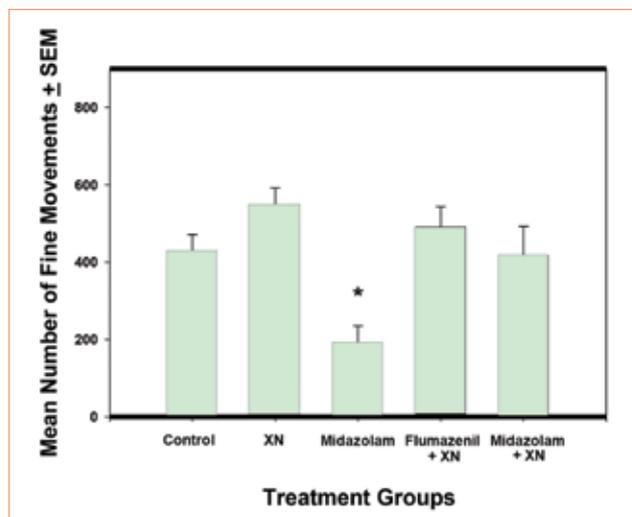* $P < .05$.

Figure 3. Fine Movements of Rats 30 Minutes After Treatment

Abbreviations: SEM, standard error of the mean; XN, xanthohumol.
* $P < .05$.

xanthohumol and midazolam (third research aim), or xanthohumol may influence the modulation of another neurotransmitter site in the central nervous system.

This study may be used to guide future research in investigating the effects of xanthohumol and hops. We recommend exploring the possibility that xanthohumol may act as a benzodiazepine $GABA_A$ partial agonist or antagonist, or may modulate another neurotransmitter system in the central nervous system. It is also important to determine the molecular site of action of xanthohumol to understand the biochemical effects of this herbal extract. We suggest that a prospective study may assist in determining whether alternative doses of benzodiazepines are required in perioperative patients who consume hops. Additionally, based on the limited scope of current research, the variety of extracts from hops is also ripe for investigation as to their site and mechanism of action, their effects on anxiety and mood, and their interactions with anesthetic medications.

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