Chapter 1

Anxiolytic and antidepressant activities of O. sanctum and C. sinensis
Introduction

The influence of environmental conditions such as stress on behavioral and cognitive processes and performance in animal models of psychiatric disorders has been widely investigated (Chrousos and Gold, 1992). Exposure to both chronic and acute stress has substantial effects on learning and memory (Sandi et al., 2005; Kleen et al., 2006). Although there is substantial literature on the effects of stress on memory from behavioral and pharmacologic perspectives, the understanding of the molecular mechanisms involved in the modulation of learning and memory by stress is still insufficient. Restraint stress is one of the most commonly employed stressors in animal models of stress-related psychopathology and has been shown to elicit complex effects on memory formation (Weiss et al., 2005). There is much interest, therefore, in understanding the mechanisms responsible for interactions among restraint stress, cognitive-emotional state, and memory. Learning and memory are complex processes involving biochemical signalling cascades that lead to change in gene expression in neurons. Stress induces a variety of autonomic, visceral, immunological and neurobehavioral responses like anxiety, depression, anorexia and activation of the hypothalamic-pituitary-adrenal axis resulting in elevated corticosterone levels, in animals and humans (Chrousos and Gold, 1992).

Anxiety is one of the most prominent psychiatric disorders related to a common stress. The elevated plus maze is a well-established and widely used animal model of anxiety-like behavior for rodents (Mechiel Korte and De Boer, 33
2003). In this paradigm, animals are faced with an approach–avoidance conflict between exploring open elevated arms and a natural tendency to hide in enclosed arms. Interestingly, a single exposure to the plus maze, in itself can modulate anxiety-like behavior exhibited by male rats during a subsequent exposure to the maze (Treit, 1993). Anxiety like behavior on the plus maze can also be modulated by prior exposure to stressors. As per the previous studies using a 21-day restraint stress paradigm have reported a significant reduction of ambulatory behavior in open-field arena and facilitated fear-conditioning in male rats (Conard et al., 1999). Even temporally restricted stressors, such as predator stress in the form of a 5 min exposure to a cat, are potent enough to trigger a state of enhanced and persistent anxiety (Adamec et al., 1999).

Depression is an incapacitating psychiatric ailment that has been estimated to affect 21% of the world population (Schechter et al., 2005). It is characterized by a pervasive low mood and loss of interest in usual activities and diminished ability to experience pleasure. Several clinical observations have suggested that stress can act as a precipitating factor in the onset of affective illness especially in case of major depression (Bidzinska, 1984). Chronic stress can induce depressive disorders, and animal stress models are widely used in pre-clinical antidepressant evaluation (Garcia, 2002). There is increasing evidence for a relationship between stress, glutamate neurotransmission, and depression (Sapolsky, 2000). Extracellular concentrations of glutamate are increased in several brain regions after exposure to behavioral stressors (Lowy et al., 1995). Correspondingly,
expression of glutamate transporters is increased after exposure to stress, potentially as a compensatory response to the increased stress-induced glutamate release (Wood et al., 2004). Additionally, a series of recent animal and human studies have demonstrated that several classes of agents that modulate glutamate neurotransmission possess antidepressant-like properties (Zarate et al., 2003). These findings support the hypothesis that excessive stress-induced glutamate release leads to a disruption in glutamate neurotransmission and cycling which contributing to the appearance of stress-related, depression-like behaviors in rodents and potentially to the pathophysiology and pathogenesis of mood disorders in humans (Sanacora et al., 2003). There is growing body of evidence showing that the chronic administration of various uncontrollable stresses, a procedure known as “chronic uncontrollable stress” is an appropriate model for the pre-clinical evaluation of antidepressants (Willner et al., 1992). Chronic unpredictable stress prolongs learned helplessness behavior and increase plasma corticosterone levels (Chen et al., 2006). It also inhibits the brain monoamine oxidase (MAO-A and MAO-B) enzyme activity (Lin et al., 2005) which may further resulted the depletion of brain monoamine levels. Numerous antidepressant compounds are now available and presumably acting via different mechanisms including serotonergic, noradrenergic and/or dopaminergic systems (Neurotransmitters). Numerous experimental and clinical studies indicate that the serotonin (5-HT) system is strongly implicated in the neural regulation of mood and several pieces of evidence have implicated the abnormalities in 5-HT neurotransmission in the
pathophysiology of depression (Wong and Licinio, 2001). Several studies indicate that an enhancement of 5-HT neurotransmission underlies the therapeutic response to various types of antidepressant treatments. Drugs affecting 5-HT neurotransmission, such as those inhibiting 5-HT reuptakes at nerve terminals or inhibiting its metabolism (monoamine oxidase inhibitors), are effective in depression (Nemeroff and Owens, 2002). The forced-swim test (FST) and the tail suspension test (TST) have been developed to evaluate depression like behaviors in mice (Porsolt et al., 1977; Steru et al., 1985). The immobility of animal in FST has been expected to reflect a state of ‘behavioral despair’. The TST also induces a state of despair in animals like that in FST. The initial activity of a rat placed in novel surroundings (open-field activity) has been taken as an indicator of its emotional state and the lack of acute activation in open field may bear some resemblance to depression. Therefore, OFT, FST and TST are widely used to evaluate the behavioral activity in animals.

Drugs obtained from natural sources are perceived to have the least risk and low side effect profiles, while having the ability to cure psychiatric disorders in much the same way as their synthetic counter parts. Ayurveda, the ancient traditional system of medicine, mentions a number of single and compound drug formulations of plant origin that are used for the treatment of psychiatric disorders. Medicinal plants, such as Bacopa monniera (Sairam et al., 2002) and Ginkgo biloba (Sakakibara et al., 2006) may be an important source of new antidepressant
drugs and the safety of nature plant extracts may also be better than that of synthetic antidepressants (Schulz, 2006).

*O. sanctum* (OS) and *C. sinensis* (CS) are commonly known as Tulsi and Green tea are well known medicinal plants. Aqueous extracts of OS and CS reportedly have antianxiety and antidepressant properties. Experimental and clinical evidence are still scarce. Therefore, this study was undertaken to evaluate the effects of these medicinal plants on anxiety and depression like behavior using EPM, OFT, TST and FST in male albino rats.

**Materials and Methods**

*O. sanctum*

Leaves of OS were collected from University campus and identified by a pharmacognist and its I.D. No. is Husain1375 which deposited for the record in A.M.U, Herbarium.

*C. sinensis*

Leaves of CS were purchased from an authorized dealer and it has also been identified by a pharmacognist and its I.D. No. is Husain 395. It is also deposited in A.M.U, Herbarium as a record.
**Extraction of OS and CS**

Briefly the shed dried powder of OS and CS were refluxed for 5 hour with double distilled water (DDW) at 100°C cooled and filtered. The solvent was removed under reduced pressure to get product (Ganasoundari et al., 1998). The yield of the extracts of OS and CS were 9% and 7% (w/w) in terms of dried starting material. The residue was stored in the refrigerator until further use.

**Animals**

Adult male albino rats (200 ± 50 gm; 8-10wk old) were obtained from Central Animal House of J.N. Medical College, A.M.U, Aligarh. The animals were kept in polypropylene cages and housed in air conditioned room and maintained on standard pellet diet and water *ad libitum*.

**Stress procedure**

The animals were restrained (Hasan, 1985) 3h/day for 6 consecutive days in a wire mashed cage (Fig 1) at a fixed time.
Fig. 1: Restraint stress
**Experimental design**

The study was approved by Institutional Animals Ethics Committee. Animals were randomized in six groups (six rats per group) and allowed to acclimatize for at least 1 week before initiating the experiments. The groups and the treatments were as follows:

- **Group I**: Control (normal saline for 6 consecutive days).
- **Group II**: Restraint stress (3h/day for 6 consecutive days).
- **Group III**: Post-administration of OS aqueous extract (for 6 consecutive days orally) following restraint stress.
- **Group IV**: Post-administration of CS aqueous extract (for 6 consecutive days orally) following restraint stress.
- **Group V**: Aqueous extract of OS (100mg/kg) for 6 consecutive days, alone.
- **Group VI**: Aqueous extract of CS (100mg/kg) for 6 consecutive days, alone.

After the treatment (restraint stress and post-administration of OS and CS) of each group the animals were carried out for behavioral tests on 7th day.

**Behavioral tests**

**Elevated plus maze test**

This test has been proposed for selective identification of anxiolytic and anxiogenic drugs. Elevated maze test was performed by the method of Montogomery (1958). The elevated plus maze consisted of two open arms and two enclosed arms, 50×40×40 cm with an open roof, assayed so that the two arms are
opposite to each other. The maze was elevated to a height of 50 cm. After treatment with each group, the rats were placed in the centre of the maze and facing one of the enclosed arms. During 5 min test period following parameters were measured: number of open arm entries and closed arm entries. Subsequently, the percentages of open arm entries and time spent on open arms were calculated from open arm entries and time spent on open arms divided by the total number of entries in both open and closed arms and time spent on open arm exploration divided by total time spent in both open and closed arms, respectively. The procedure was conducted in sound attenuated room.

Open field test

Locomotor activity was quantified for 5 min in an open field. It was consisted of a square arena 96×96 cm with 60-cm high walls. The walls and the floor were painted white. The floor was divided in to 16 squares by parallel and intersecting color lines (Bhattacharya and Satyan, 1997). Four squares defined as the centre and the 12 squares along the walls as the periphery. Rats were placed in the centre of the open field and (a) latency, (b) ambulation and (c) rearing were observed during a 5 min exposure period for both control and treated animals.

Tail suspension test

This test was performed as described by Steru et al. (1985). A short piece of paper adhesive tape (about 6 cm) was attached along half the length of the tail (about 3
cm). The free end of the tape was attached to a 30 cm long rigid tape which hung from a horizontal bar clamped to a heavy laboratory support stand. Suspended animals were surrounded by a white wooden enclosure (40 cm high, 40 cm wide and 40 cm deep) such that the rat's head was about 20 cm above the floor. For testing, each rat was suspended by its tail and observed for 6 min. an observer scored the total duration of a passive, "dead weight" hanging (immobility) between the periods of wriggling of the animal to avoid aversive situation.

**Forced swim test**

The test was performed according to a modification suggested by Lucki of the traditional method (Porsolt et al., 1977). The apparatus consisted of a transparent cylinder (50 cm high × 20 cm wide) filled to 30 cm depth with water at room temperature. The water depth was adjusted so that the animals must swim or float without their hind limbs or tail touching the bottom. The duration of immobility was recorded during the last 5 min of the 6-min test session (Bhatwadekar et al., 1991). A rat was judged to be immobile when it floated in an upright position and making only small movements to keep its head above water.
Fig. 2: Elevated plus maze test

Fig. 3: Open field behaviour test
Fig. 4: Tail suspension test

Fig. 5: Forced swim test
Statistical analysis

Results are expressed as Mean±SEM. Behavioral data were analyzed using student’s t-test. Significance level was chosen at $P < 0.05$, $P < 0.01$ and $P < 0.001$. All statistical analyses were carried out by using SPSS for Windows (SPSS 10.0).

Results

Elevated plus maze test

Analysis of elevated plus maze data revealed that restraint stress (3h/day for 6 days) induced a significant reduction in % open arm entries (50%) and % time spent in open arms (63%) as compared to control ($P < 0.05$). Post-treatment of OS and CS aqueous extracts (100 mg/kg for 6 days) reversed the restraint stress-induced changes in both open arm entries and % time spent in open arms. OS and CS significantly increased the % number of entries (54%, 38%) respectively as compared to stress group ($P < 0.01$, $P < 0.05$). % time spent in open arms was also increased (65%, 53%) significantly with the treatment of OS and CS. There was no significant change when OS and CS were given alone (Table 1, Fig 6).

Open field test

Restraint stress induced a marked increase in the latency (48%), decrease in ambulation (38%) and rearing (59%) significantly as compared to their respective control group ($P < 0.05$). Post-treatment of OS and CS attenuated the restraint stress effects on the open field behavior i.e. latency of entry was decreased (52%,
25%), and both ambulation (41%, 33%) and rearing (64%, 49%) activity were increased respectively as compared to stress ($P < 0.01$, $P < 0.05$). OS and CS per se group did not show any significant change (Table 2, Fig 7).

**Tail suspension test**

Restraint stressed rats exhibited significant increase in immobility period (46%) as compared to control animals ($P < 0.001$). Post-administration of OS and CS reversed the increase in immobility period in stressed rats. When OS and CS were administered the significant protection was observed in TST. OS decreased the 48% immobility while 40% by CS ($P < 0.05$). Both of these plants did not show any significant change as compared to control (Table 3, Fig 8).

**Forced swimming test**

Restraint stress significantly increased the immobility (53%) in the FST as compared to control ($P < 0.001$). OS caused a 57% reduction in immobility while CS 49% as compared to their respective stress group ($P < 0.05$). There was no significant effect on the immobility when OS and CS were given alone (Table 4, Fig 9).
Table 1: Protective effect of *O. sanctum* and *C. sinensis* on restraint stress-induced alterations in elevated plus-maze test (EPM).

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Open arm entries (Mean ± S.E)</th>
<th>% Open arm time (Mean ± S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.2 ± 3.4</td>
<td>17.3 ± 3.6</td>
</tr>
<tr>
<td>RS</td>
<td>12.5 ± 4.3*</td>
<td>6.4 ± 2.4*</td>
</tr>
<tr>
<td>RS + O.S</td>
<td>27.4 ± 2.0**</td>
<td>18.5 ± 2.8**</td>
</tr>
<tr>
<td>OS</td>
<td>26.7 ± 2.4</td>
<td>20.4 ± 2.6</td>
</tr>
<tr>
<td>RS + C.S</td>
<td>20.3 ± 3.1**</td>
<td>13.6 ± 3.2^a</td>
</tr>
<tr>
<td>CS</td>
<td>22.3 ± 3.6</td>
<td>15.4 ± 1.4</td>
</tr>
</tbody>
</table>

Data show the mean of ± S.E of six animals.

*P < 0.05. Statistically significant as compared to control group.

^P < 0.05, **P < 0.01. Statistically significant as compared to RS group.
Fig. 6: Bar diagram showing the alteration of % Open arm entries and % Time spent in EPM following restraint stress (3h/day for 6 days) and ameliorative action of *O. sanctum* and *C. sinensis* (100mg/kg/day for 6 days) on *Rattus norvegicus*. 
Table 2: Protective effect of *O. sanctum* and *C. sinensis* on restraint stress-induced alterations in open field behavior test (OFT).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Latency</th>
<th>Ambulation</th>
<th>Rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5 ± 0.7</td>
<td>52.7 ± 5.2</td>
<td>25.2 ± 2.5</td>
</tr>
<tr>
<td>RS</td>
<td>4.8 ± 0.3*</td>
<td>32.6 ± 4.6*</td>
<td>10.4 ± 1.3*</td>
</tr>
<tr>
<td>RS + O.S</td>
<td>2.3 ± 0.5a</td>
<td>55.4 ± 3.1**</td>
<td>28.7 ± 3.0**</td>
</tr>
<tr>
<td>OS</td>
<td>2.7 ± 0.4</td>
<td>53.6 ± 2.8</td>
<td>26.4 ± 2.2</td>
</tr>
<tr>
<td>RS + C.S</td>
<td>3.6 ± 0.2**</td>
<td>48.6 ± 4.2a</td>
<td>20.3 ± 1.9**</td>
</tr>
<tr>
<td>CS</td>
<td>2.8 ± 0.3</td>
<td>53.8 ± 3.6</td>
<td>27.6 ± 3.2</td>
</tr>
</tbody>
</table>

Data show the mean of ± S.E of six animals  
*P < 0.05. Statistically significant as compared to control group.  
*aP < 0.05, **P < 0.01. Statistically significant as compared to RS group.
Fig. 7: Bar diagram showing the alteration of Latency, Ambulation and Rearing in OFT following restraint stress (3h/day for 6 days) and ameliorative action of *O. sanctum* and *C. sinensis* (100mg/kg/day for 6 days) on *Rattus norvegicus*. 
Table 3: Protective effect of *O. sanctum* and *C. sinensis* on restraint stress-induced alterations in tail suspension test (TST).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Immobility time (in seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.73 ± 7.3</td>
</tr>
<tr>
<td>RS</td>
<td>150.64 ± 4.1*</td>
</tr>
<tr>
<td>RS + O.S</td>
<td>78.84 ± 5.7**</td>
</tr>
<tr>
<td>OS</td>
<td>82.36 ± 6.4</td>
</tr>
<tr>
<td>RS + CS</td>
<td>90.34 ± 6.7**</td>
</tr>
<tr>
<td>CS</td>
<td>85.72 ± 4.6</td>
</tr>
</tbody>
</table>

Data show the mean of ± S.E of six animals.

*P < 0.001. Statistically significant as compared to control group.

**P < 0.05. Statistically significant as compared to RS group.
Fig. 8: Bar diagram showing the alteration of Immobility time in TST following restraint stress (3h/day for 6 days) and ameliorative action of *O. sanctum* and *C. sinensis* (100mg/kg/day for 6 days) on *Rattus norvegicus*. 
Table 4: Protective effect of *O. sanctum* and *C. sinensis* on restraint stress-induced alterations in forced swim test (FST).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Duration of Immobility (in seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60.83 ± 12.4</td>
</tr>
<tr>
<td>RS</td>
<td>130.5 ± 15.2*</td>
</tr>
<tr>
<td>RS + O.S</td>
<td>55.62 ± 10.4**</td>
</tr>
<tr>
<td>OS</td>
<td>58.73 ± 9.2</td>
</tr>
<tr>
<td>RS + C.S</td>
<td>66.73 ± 10.2**</td>
</tr>
<tr>
<td>CS</td>
<td>63.21 ± 11.5</td>
</tr>
</tbody>
</table>

Data show the mean of ± S.E of six animals.

*P < 0.001. Statistically significant as compared to control group.

**P < 0.05. Statistically significant as compared to RS group.
Fig. 9: Bar diagram showing the alteration of Immobility time in FST following restraint stress (3h/day for 6 days) and ameliorative action of *O. sanctum* and *C. sinensis* (100mg/kg/day for 6 days) on *Rattus norvegicus*. 
Discussion

The present study investigated the effects of OS and CS on restraint stress induced anxiety and depression like behavior such as EPM, OFT, TST and FST.

Emotional stressors like restraint stress (RS) can influence the neurobehavioral profile of the organism and complex interactive mechanisms have been proposed for these effects. Many studies have indicated changes in behavioral and biochemical characteristics in depressed patients. In the present study we found that exposure to RS (3h/day for 6 days) rats appeared to have behavioral deficits including suppressed the activity of EPM, OFT and increased immobility in TST and FST. RS-induced alterations reversed with post treatment of aqueous extracts of OS and CS.

Both EPM and OFT have been used effectively to assess neurobehavioral profile of animals under the influence of anxiogenic/anxiolytic agents (Carobrez and Bertoglio, 2005). EPM increased aversion of open arms are indicative of enhanced anxiety state and our results indicated that sub-acute restraint stress caused reduction in the % number of entries and % time spent in open arms in EPM activity. Similarly, in the OFT exposure to RS-induced behavioral alterations was evidenced by increase in latency and decrease in ambulation and rearing. These are the indications of high level of fear or anxiety. Our results are in agreement with earlier studies (Masood et al., 2003; Chakraborti et al., 2007). Vyas et al. (2003) reported that chronic immobilization stress (CIS) elicits enhanced anxiety in male rats. Antioxidant treatment has shown modulating
effects on brain free radicals in restrained rats (Zaidi et al., 2003). Aqueous extracts of OS and CS were significantly increased the number of open arm entries and time spent in open arms. In OFT OS and CS decreased latency, increased ambulation and rearing significantly. These behavioral changes are suggestive of decreased fear or anxiety. So, both of these plants have shown anxiolytic effects against RS. Our results are strongly supported by the study of Gopala Krishna et al. (2006) who has reported antianxiety activity of NR-ANX-C, a polyherbal preparation, containing aqueous extracts of Withania somnifera and Shilajit and alcoholic extracts of OS and CS. OS is shown to have cortisol sparing immunostimulant and antioxidant activities. This cortisol sparing immunomodulatory activity of OS may also contribute to the behavioral disinhibitory activity (Bhargava and Singh, 1981; Sen et al., 1992). Earlier studies have shown that CS extract contain many of polyphenolic antioxidants such as catechins, epicatechin and epigallocatechin gallate. Epicatechin, one of its polyphenolic constituent has been found to enhance learning and memory ability in mice (Matsuoka et al., 1995). Epigallocatechin gallate markedly increased protein kinase C in the membrane and the cytosolic fractions of mice hippocampus the learning site of brain (Levites et al., 2003). Oral administration of the aqueous extract of M. officinalis was enhanced the exploratory behavior of mice in the EPM and OFT and induced sedation (Coleta et al., 2001). So, our results are strongly supported the previous studies.
Both FST and TST are widely used to screen new antidepressant drugs. These tests are quite sensitive and relatively specific to all major classes of antidepressant drugs including tricyclics, 5-HT-specific reuptake inhibitors, MAO inhibitors and atypicals (Porsolt et al., 1977; Steru et al., 1985). In FST, mice are forced to swim in a restricted space from which they cannot escape, and are induced to a characteristic behavior of immobility. This behavior, reflecting a state of despair, is reduced by several agents which are therapeutically effective in human depression. This immobility referred to as behavioral despair in animals, is claimed to reproduce a condition similar to human depression (Steru et al., 1985; Willner, 1984). There are many herbal extracts such as *A. venetum*, *A. catechu*, *B. monniera*, *C. sympodialis* and *R. stricta* which have shown antidepressant activity (Kim et al., 2000; Butterweck et al., 2001; Sairam et al., 2002). As a natural antidepressant agent, *H. perforatum* is one of the well investigated medicinal plants (Butterweck, 2003). According to our results, OS and CS given orally are effective in producing significant antidepressant-like effects when assessed in FST and in TST. The precise mechanisms by which OS and CS produced antidepressant-like effects are not completely understood. There is a complex relationship among stressful situations, mind and body's reaction to stress and the onset of clinical depression. Some stress-provoked disturbances seem to be associated with the pathophysiology of depression (Kioukia-Fougia et al., 2002). Various antidepressant drugs, either by inhibiting MAO-enzyme or by inhibiting reuptake mechanism, increase the central monoamine levels or reverse the stress-
induced depressive-like behavior. According to Samson et al. (2006) noise stress increased the level of neurotransmitters (dopamine, serotonin) significantly in different parts of brain like cerebral cortex, cerebellum, hypothalamus, hippocampus (memory center), and corpus striatum. OS prevented increase in neurotransmitter levels of stress treated group. In another study swimming and gravitational stress decreased the level of brain adrenaline (A), noradrenaline (NA) while increased dopamine (DA) and serotonin (5-HT) levels. MAO level was found to be decreased after stress. These stress-induced alterations reversed to normal level with the treatment of OS (Singh et al., 1991). CS and its phenolic components catechins and epigallocatechin gallate, have been found to be effective at inhibiting MAO level. Theanine an amino acid found in CS has also been found to have beneficial effects by rasing the levels of serotonin and dopamine in various important brain regions, particularly the hypothalamus, hippocampus and striatum (Yokogoshi et al., 1998). These suggest that OS and CS might produce antidepressant-like effect by interaction with adrenoceptors, 5-HT and dopamine receptors, and monoamine oxidase, thereby increasing or decreasing the levels of NA, 5-HT and dopamine in the brains of rats. Several herbal preparations that have antidepressant potential also possess antioxidant effects. Antioxidant action may have a role in the mechanisms responsible for antidepressant activity of some herbal constituents (Zhang-Jin Zhang, 2004). Both OS and CS are antioxidant medicinal plants. So antidepressant activity may be correlated to antioxidant activity of these plants.
Conclusion

In conclusion, OS and CS exerted anxiolytic and antidepressant-like effect in restraint stress-induced anxiety and depression model in rats and these effects may be mediated by the central monoaminergic neurotransmitter system (5-HT and dopamine). In the entire behavioral test OS shows higher protection than CS. So, we concluded that OS is more protective as compared to CS to reduce anxiety and depression. Further, this finding provides a scientific rationale for the co-administration of OS and CS which may act as a useful and potent combination in the treatment of anxiety and depressive disorders.