4. MATERIALS AND METHODS
4.1 Drugs


4.2 Plant material and preparation of the extract

Fruits of black pepper (*Piper nigrum*, family Piperaceae) were purchased from local market (Figure 21). The botanical identification of the fruits was done by Dr. Dhabe, Herbarium incharge Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (M.S.), India, where a voucher specimen has been deposited. After collection, the fruits were ground to coarse powder. 200 gm of the powdered fruit was boiled with 2 lit of distilled water in a conical flask for 30 min and the liquid was decanted. The resultant filtrate was evaporated to dryness in the oven at 40 °C. The dried aqueous black pepper extract (ABPE) was reconstituted in distilled water.

4.3 Isolation of Piperine

10 g of ground black pepper was placed in a 150 ml round bottom flask with 150 ml of 95% ethanol and boiling chips and heated at reflux for 2 hr. The mixture was filtered by suction filtration and filtrate was concentrated to 10 – 15 ml by simple distillation. The concentrated black pepper extract was added to 10 ml of a 10% solution of KOH in 95 % ethanol contained in a 125 ml Erlenmeyer flask. The resulting solution was heated with the drop wise addition of water, a yellow precipitate was formed. Water was added until no more solids to appear to form and the mixture was allowed to stand overnight. Solids were collected by suction filtration and recrystallised it with 20 ml of acetone (Agarwal et al., 2007). Finally, the yellow coloured needle shaped precipitates of piperine was collected (Figure 19).
4.4 Characterisation of Black pepper extract:

Characterisation of black pepper extract was done by chemical test, melting point, UV spectroscopy analysis and thin layer chromatography (TLC). For a TLC a small portion of the purified crystals were transferred to a small vial and dissolved it in methanol (approx. 1 mg in 1 ml). Ready to use TLC plates were used for spotting of samples. Sample of the crude extract remaining from the extraction procedure and the sample prepared from the purified piperine was placed with the aid of capillary tube and developed with toluene:EtOAc 7:3 and stain in an iodine chamber. The purity of the product is checked by measuring its UV spectrum (Harborne, 2005, Agarwal et al., 2007, Kanki et al., 2008).

4.5 Composition of Mebarid, Enterocin and Kutajarishta.

Each 10 ml of Mebarid contains i) Ajmoda (100 mg), ii) Bael (100 mg), iii) Lodhara (100 mg), iv) Dadim (100 mg), v) Badishep (100 mg), vi) Daruhalad (100 mg), vii) Jaiphal (50 mg), viii) Sunth (50 mg), ix) Ativish (50 mg), x) Kuda (50 mg), xi) Sugar (q.s.).

Each 4 ml of Enterocin contains i) Vidangphal (1250 mg), ii) Daruhaladi chaal (1000 mg), iii) Dhaiphool (500 mg), iv) Kuda chaal (500 mg), v) Shodhit geirik pashan (500 mg), vi) Mustamool (500 mg), vii) Lodhara chaal (500 mg), viii) Ativishmool (250 mg), ix) Soonthimool (250 mg), x) Saindhav (10 mg), xi) Sanchal (10 mg), xii) Syrup base (q.s.).

Each 10 ml of Kutajarishta contains i) Kutaja (3 gm), ii) Draksa (1.5 gm), iii) Madhuka Puspa (300 mg), iv) Gambhari (300 mg), v) Dhataki (600 mg) vi) Guda (3 gm), vii) Asav Base (q.s.).

4.6 Animals

Swiss albino mice of either sex, weighing 20 – 25 gm obtained from VIPER, Pune, were used for the experiments. They were kept in standard environmental condition, fed standard food and water ad libitum. All experiments were performed after an overnight fast.

Guinea pigs (300 – 400 gm each) were obtained from VIPER, Pune. They were fed with commercially available standard feed. The animals were housed under standard condition. Water was provided ad libitum. The Institutional Animal Ethical Committee of
Government College of Pharmacy, Aurangabad, Maharashtra, India (GCPA/IAEC/2011/235, 11/03/2011), approved the study.

4.7 Acute toxicity study

Initially the antidiarrhoeal herbal formulations like Mebarid, Enterocin, Kutajarishta, aqueous black pepper extract and piperine were studied for acute oral toxicity as per revised OECD guidelines number 423. Mebarid, Enterocin and Kutajarishta were devoid of any toxicity up to 20 ml/kg in albino mice by oral route. Hence for further studies 2.5 to 10 ml/kg doses of these formulations were used. Aqueous black pepper extract were devoid of any toxicity up to 2000 mg/kg in albino mice by oral route. Hence for further studies doses of 75 to 300 mg/kg of aqueous black pepper extracts were used. Piperine was used for the study at the dose of 20 mg/kg because it has not shown any toxicity up to 50 mg/kg.

4.8 Experimental procedure for antidiarrhoeal activity

4.8.1 Castor oil induced diarrhoea

4.8.1.1 Effect of Mebarid, Enterocin, Kutajarishta, aqueous Black pepper extract, Piperine and Loperamide on castor oil induced diarrhoea.

Groups of six mice each were treated as outlined below:
Group 1 (Control group): Distilled water 10 ml/kg, p.o.,
Group 2 (Standard group): Loperamide 2 mg/kg, p.o.,
Group 3 (Test group): Mebarid 2.5, 5, 10 ml/kg, p.o.,
Group 4 (Test group): Enterocin 2.5, 5, 10 ml/kg, p.o.,
Group 5 (Test group): Kutajarishta 2.5, 5, 10 ml/kg, p.o.,
Group 6 (Test group): ABPE 75, 150, 300 mg/kg, p.o.
Group 7 (Test group): Piperine 20 mg/kg, p.o.

After 30 min, castor oil (0.2 ml/mouse) was administered to each mouse. The animals were then placed under separate glass funnels, with the floor lined with blotting paper, for observation for 4 h. The parameters observed were: onset of diarrhoea, total weight of faecal output, total weight of wet faeces, total number of faecal output, and number of wet faeces (Adeyemi et al., 2009).
4.8.1.2 Effect of Glibenclamide, Isosorbide dinitrate and Yohimbine on antidiarrhoeal activity of Mebarid, Enterocin, Kutajarishta and aqueous Black pepper extract on castor oil induced diarrhoea.

The same procedure as in the castor oil induced diarrhoea was followed except the treatment. Groups of six mice each for Mebarid (2.5 ml/kg, p.o.), Enterocin (2.5 ml/kg, p.o.), Kutajarishta (2.5 ml/kg, p.o.) and ABPE (300 mg/kg, p.o.), were treated as outlined below (Adeyemi et al., 2009, Flavia et al., 1999, Mbagwu et al., 2008):

Group 1: Glibenclamide 1 mg/kg, p.o. (given 30 min prior to the administration of Mebarid, Enterocin, Kutajarishta and ABPE),

Group 2: Isosorbide dinitrate 150 mg/kg, p.o. (given 30 min prior to the administration of Mebarid, Enterocin, Kutajarishta and ABPE),

Group 3: Yohimbine 1 mg/kg, s.c. (given 30 min prior to the administration of Mebarid, Enterocin, Kutajarishta and ABPE).

4.8.1.3 Effect of aqueous Black pepper extract on antidiarrhoeal activity of Mebarid, Enterocin, and Kutajarishta on castor oil induced diarrhoea.

The procedure remained same as mentioned in the castor oil induced diarrhoea except the treatment. Groups of six mice each for Mebarid, Enterocin, and Kutajarishta were treated as mentioned below (Adeyemi et al., 2009):

Group 1: ABPE 300 mg/kg, p.o. given with Mebarid 2.5 ml/kg, p.o.,
Group 2: ABPE 300 mg/kg, p.o. given with Enterocin 2.5 ml/kg, p.o.,
Group 3: ABPE 300 mg/kg, p.o. given with Kutajarishta 2.5 ml/kg, p.o.

4.8.2 Magnesium sulfate induced diarrhoea

4.8.2.1 Effect of Mebarid, Enterocin, Kutajarishta, aqueous Black pepper extract, Piperine and Loperamide on magnesium sulfate induced diarrhoea.

A similar protocol as for castor oil induced diarrhoea was followed (Afroz et al., 2006). Magnesium sulfate was given in the dose of 2 g/kg to the animals 30 min after pre-treatment with (Rouf et al., 2007, Sairam et al., 2003):

Group 1 (Control group): Distilled water 10 ml/kg, p.o.,
Group 2 (Standard group): Loperamide 2 mg/kg, p.o.,
Group 3 (Test group): Mebarid 2.5, 5, 10 ml/kg, p.o.,
Group 4 (Test group): Enterocin 2.5, 5, 10 ml/kg, p.o.,
Group 5 (Test group): Kutajarishta 2.5, 5, 10 ml/kg, p.o.,
Group 6 (Test group): ABPE 75, 150, 300 mg/kg, p.o.
Group 7 (Test group): Piperine 20 mg/kg, p.o.

4.8.2.2 Effect of aqueous Black pepper extract on antidiarrhoal activity of Mebarid, Enterocin, and Kutajarishta on magnesium sulfate induced diarrhoea.

The procedure was similar to magnesium sulfate induced diarrhoea except the treatment (Afroz et al., 2006, Rouf et al., 2007, Uddin et al. 2005). Groups of six mice each for Mebarid, Enterocin, and Kutajarishta were treated as outlined below:

Group 1: ABPE 300 mg/kg, p.o. given with Mebarid 2.5 ml/kg, p.o.,
Group 2: ABPE 300 mg/kg, p.o. given with Enterocin 2.5 ml/kg, p.o.,
Group 3: ABPE 300 mg/kg, p.o. given with Kutajarishta 2.5 ml/kg, p.o.

4.8.3. Gastrointestinal motility by charcoal meal

4.8.3.1. Effect of Mebarid, Enterocin, Kutajarishta, aqueous Black pepper extract, Piperine and Atropine sulphate on castor oil induced gastrointestinal motility.

Six mice were allotted to different groups. Treatment was then carried out as outlined below:

Group 1 (Normal group): Distilled water 10 ml/kg, p.o.,
Group 2 (Control group): Distilled water 10 ml/kg, p.o.,
Group 3 (Standard group): Atropine sulphate 5 mg/kg, i.p.,
Group 4 (Test group): Mebarid 2.5, 5, 10 ml/kg, p.o.,
Group 5 (Test group): Enterocin 2.5, 5, 10 ml/kg, p.o.,
Group 6 (Test group): Kutajarishta 2.5, 5, 10 ml/kg, p.o.,
Group 7 (Test group): ABPE 75, 150, 300 mg/kg, p.o.,
Group 8 (Test group): Piperine 20 mg/kg, p.o.

After 30 min treatment, each animal was given castor oil (0.2 ml/mouse, p.o.) except Group 1 (Normal Group). Each animal was given orally 0.2 ml of charcoal meal (3% charcoal in 5% gum acacia), 30 min after castor oil administration. Animals were
sacrificed 30 min after administration of charcoal meal and the small intestine immediately isolated. Peristaltic index for each mouse was expressed as percentage of the distance travelled by the charcoal meal relative to the total length of the small intestine (Adeyemi et al., 2008, Adeyemi et al., 2009, Vareishang).

4.8.3.2. Effect of aqueous Black pepper extract in combination with Mebarid, Enterocin and Kutajarishta on castor oil induced gastrointestinal motility.

Mice (six) were used for different groups of Mebarid, Enterocin, and Kutajarishta. Treatment was then carried out as outlined below:

Group 1: ABPE 300 mg/kg, p.o. given with Mebarid 2.5 ml/kg, p.o.,
Group 2: ABPE 300 mg/kg, p.o. given with Enterocin 2.5 ml/kg, p.o.,
Group 3: ABPE 300 mg/kg, p.o. given with Kutajarishta 2.5 ml/kg, p.o.

Procedure followed was same as given in the gastrointestinal motility except the treatment mentioned above (Adeyemi et al., 2006, ).

4.8.4. Small intestinal secretions

4.8.4.1 Effect of Mebarid, Enterocin, Kutajarishta, aqueous Black pepper extract, Piperine and Chlorpromazine on castor oil induced small intestinal secretions.

Effect of Mebarid, Enterocin, Kutajarishta and ABPE on intestinal secretion was indirectly studied by enteropooling assay. Six mice were allotted to different groups. Treatment was then carried out as outlined below (Mujumdar et al., 2005, Wendel et al., 2008):

Group 1 (Normal group): Distilled water 10 ml/kg, p.o.,
Group 2 (Control group): Distilled water 10 ml/kg, p.o.,
Group 3 (Standard group): Chlorpromazine 30 mg/kg i.p.,
Group 4 (Test group): Mebarid 2.5, 5, 10 ml/kg, p.o.,
Group 5 (Test group): Enterocin 2.5, 5, 10 ml/kg, p.o.,
Group 6 (Test group): Kutajarishta 2.5, 5, 10 ml/kg, p.o.,
Group 7 (Test group): ABPE 75, 150, 300 mg/kg, p.o.,
Group 8 (Test group): Piperine 20 mg/kg, p.o.

Castor oil (0.2 ml/mouse) was administered to each mouse except Group 1 (Normal Group) after 30 min of above treatment. The mice were sacrificed 30 min after castor oil administration and the entire small intestine from each animal was weighed and their
group average was calculated. The difference in the weight of intestine in control and castor oil treated group was considered as the castor oil induced accumulation of intestinal fluid (Mujumdar et al., 2005, Mbagwu et al., 2008).

4.8.4.2. Effect of aqueous Black pepper extract in combination with Mebarid, Enterocin, and Kutajarishta on castor oil induced small intestinal secretions.

The same procedure as in the small intestinal secretion was followed except the treatment (Mujumdar et al., 2005). Group of six mice were allotted each for Mebarid, Enterocin, and Kutajarishta. Treatment was then carried out as outlined below:

Group 1: ABPE 300 mg/kg, p.o. given with Mebarid 2.5 ml/kg, p.o.,
Group 2: ABPE 300 mg/kg, p.o. given with Enterocin 2.5 ml/kg, p.o.,
Group 3: ABPE 300 mg/kg, p.o. given with Kutajarishta 2.5 ml/kg, p.o.

4.8.5 Guinea pig ileum preparations.

4.8.5.1 Effect of Mebarid, Enterocin, Kutajarishta and aqueous Black pepper extract on stimulant effect of Acetyl choline, Nicotine, Histamine and Calcium in isolated guinea pig ileum.

Guinea pigs were killed by a cervical blow using an iron rod. The abdomen was opened and the caecum was identified. It was lifted forward and the ileum was found to be joined at its back. A piece of ileum was removed, cleaned of fat and adhering connective tissues such as mesenteries and was placed in a petri dish containing Tyrode solution. A thread was attached to the top to serve as a marker. The perfusion fluid in petri dish was aerated and debris inside the lumen was washed gently with pipette. The mesenteric membrane was trimmed for a length of ileum of approximately 2 cm. Two threads were tied to the upper and lower portion of the gut. The thread tied to the lower portion was attached to the hook of the air-delivery tube inside the bottom of the chamber, in a water jacketed organ bath containing 10 ml Tyrode solution (composition in mM: NaCl 136.89, KCl 2.68, MgCl$_2$ 1.05, CaCl$_2$ 1.36, NaH$_2$PO$_4$ 0.32, NaHCO$_3$ 11.90 and glucose 5.55) and the thread tied to the upper portion of gut was attached to the force displacement transducer (Arul et al., 2004). Tissues were mounted under an initial load of 0.5 g and allowed to equilibrate for 30 min. before the addition of any drug. The experiments were performed at 37 °C and bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Normal rhythmic motility was recorded on a student’s physiograph (Bio-Device, Ambala – 134003) (Jing Hu et al.,
The effect of Mebarid (0.2 ml/ml), Enterocin (0.2 ml/ml), Kutajarishta (0.2 ml/ml) and aqueous extract of black pepper (3 mg/ml) was tested on spontaneous contractions of guinea pig ileum induced by acetyl choline (1µM), nicotine (2 µg/ml), histamine (1µg/ml) and calcium (25 µg/ml).

4.9 Preliminary Phytochemical Analysis

Plants may be considered as biosynthetic laboratory for multitude of compounds like alkaloids, glycosides, volatile oils, tannins, saponins, flavonoids and etc. These compounds are termed as secondary metabolites and are responsible for therapeutic effects.

To check the presence or absence of primary and secondary metabolites, all the formulations and extracts were subjected to battery of chemical tests (Khandelwal, 2007, Kokate et al., 2007).

4.9.1 Test for carbohydrate

4.9.1.1 Molish’s test

To the test solution added few drops of alcoholic α-naphthol, then added few drops of concentrated sulphuric acid through sides of test tube, purple to violet colour ring appeared at the junction.

4.9.2 Test for steroids and triterpenoids

4.9.2.1 Libermann-Burchard test

The drug is treated with few drops of acetic anhydride, boiled and cooled. Then added concentrated sulphuric acid from side of the test tube, brown ring is formed at the junction of two layers and upper layer turns green which showed the presence of steroids and formation of deep red colour indicated the presence of triterpenoids.
4.9.2.2 Salkowaski test

The drug is treated with few drops of concentrated sulphuric acid, red colour at upper layer indicated presence of steroids and formation of yellow coloured lower layer indicated presence of triterpenoids.

**4.9.3 Test for alkaloids**

To the test drug, added dilute hydrochloric acid, shaked well and filtered. With filtrate following tests were performed:

4.9.3.1 Dragendorff’s test

To 2 – 3 ml filtrate added few drops of Dragendorff’s reagent (potassium bismuth iodide solution) orange brown precipitate is formed.

4.9.3.2 Wagner’s test

To 2 – 3 ml filtrate added few drops of Wagner’s reagent (iodine-potassium iodide solution) gave reddish brown precipitate.

4.9.3.3 Hager’s test

To 2 – 3 ml filtrate added few drops of Hager’s reagent (saturated solution of picric acid) gave yellow precipitate.

**4.9.4 Test for glycosides**

4.9.4.1 Legal’s test (for cardiac glycosides)

The test solution was treated with pyridine and added alkaline sodium nitroprusside solution, blood red colour appeared.

4.9.4.2 Borntrager’s test (for anthraquinone glycosides)

To 3 ml test solution added dilute $\text{H}_2\text{SO}_4$. Boiled and filtered. To cold filtrate, added equal volume of benzene or chloroform. Shaked well, separated the organic solvent and added ammonia. Ammoniacal layers turned pink or red.
4.9.4.3 Modified Borntrager’s test (for anthraquinone glycosides)

To 5 ml test solution added 5 ml 5% ferric chloride and 5 ml dilute HCL. Heated for 5 min in boiling water bath. Cooled and added benzene or any organic solvent. Shaked well, separated organic layer, added equal volume of dilute ammonia. Ammoniacal layers showed pinkish red colour.

4.9.4.4 Foam test (for saponins glycosides)

Placed 2 ml solution of drug in water in a test tube, shaked well, stable froth (foam) is formed.

4.9.4.5 Shinoda test (for flavonoids)

To the test solution added few magnesium turnings and concentrated HCl drop wise. Pink colour appeared.

4.9.4.6 Lead acetate test (for flavonoids)

To the test drug added lead acetate solution. Yellow colour precipitate is formed.

4.9.5. Test for tannins

4.9.5.1. Ferric chloride (FeCl₃) test

To 2 – 3 ml of test solution added few drops of FeCl₃. Deep blue – black colour is formed

4.9.5.2. Potassium dichromate test

To 2 – 3 ml of test solution added few drops of potassium dichromate. Red precipitate is formed.

4.9.5.3. Gelatin test

To 2 – 3 ml of test solution added few drops of gelatin solution. White precipitate is formed.

4.9.6. Test for piperine

4.9.6.1. Wagner’s reagent test
Wagner’s reagent added to an alcoholic solution of piperine formed bluish needle like crystals.

4.9.6.2. Sulphuric acid test

Piperine reacted with a few drops of concentrated sulphuric acid, yielded a distinct red colouration

4.10 Statistics

The results of all experiments were reported as mean ± S.E.M. Statistical analysis was carried out using Student’s ‘t’-test. A level of significance of $P < 0.05$ was regarded as statistically significant.