FINAL REPORT

UGC Reference no: 42-689/2013 (SR) from 01.04.2013 to 31.03.2017

TITLE OF THE RESEARCH PROJECT

"EVALUATION OF GUGGULIPID EXTRACT IN ANIMAL MODEL OF FOCAL CEREBRAL ISCHEMIA IN RATS"

ज्ञान-विज्ञान विमुक्तये

THE SECRETARY

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI-110002

Submitted by:

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New Delhi-110002

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"EVALUATION OF GUGGULIPID EXTRACT IN ANIMAL MODEL OF FOCAL CEREBRAL ISCHEMIA IN RATS"

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1. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR:

DR. MOHAMMAD. AKHTAR

2. NAME AND ADDRESS OF THE INSTITUTION

DEPARTMENT OF PHARMACOLOGY
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NEW DELHI

3. TITLE OF THE PROJECT **"Evaluation of guggulipid extract in animal model of focal cerebral ischemia in rats"**

4**. OBJECTIVES OF THE PROJECT:**

The present study evaluated the effects of gugulipid extract in focal cerebral ischemia as pretreatment and post treatment set up with following objectives:

- Preparation of gugulipid extract
- Induction of cerebral ischemia by middle cerebral artery occlusion (MCAO) model in rats
- High fat diet induced apoptosis of hypothalamic neurons
- Effect of gugulipid extract on high fat diet induced apoptosis of hypothalamic neurons and on the MCAO model followed by behavioural tests and estimation of markers of oxidative stress in rat's brain.
- Comparison of protective effects of gugulipid extract alone and in combination with available medicine for stroke.

5. WHETHER OBJECTIVES WERE ACHIEVED (GIVE DETAILS):

The objectives of the research work were achieved. Guggulipid extract was preapred and standardized. Determination of percentage yield was also carried out. Preliminary phytochemical screening of Guggulipid extract showed the presence of guggulsterone, diterpenoids, lipids steroid and phenolic and flavonoid components. Determinations of physicochemical properties were also performed followed by HPTLC finger printing.

Cerebral ischemia was induced using middle cerebral artery occlusion model (MCAO) in rats. Various behavioral parameters were assessed with the final biochemical estimations which include TBARS, GSH, SOD, Catalase, NO, $Na^{+}K^{+}$ ATPase, acetylcholinesterase. The recording of cerebral blood flow using laser Doppler blood flow meter and finally histopathological estimations were also performed.

6. ACHIEVEMENTS FROM THE PROJECT:

The project's aims and objectives were completed well and the positive outcome after reserach work was achieved and was explained in work done completed part.

a. One research scholar was trained

b. The research scholar was registered for Ph.D. and Ph.D degree was awarded.

c. Two research papers were published.

Our results showed that that chronic pre-treatment of guggulipid may be helpful in reducing the symptoms of cerebral ischemia in rats. Neuroprotective actions of guggulipd observed in the present study were due to anti-inflammatory, antioxidant, and antithrombotic effects that may be neuroprotective during cerebral ischemia and reperfusion.

7. SUMMARY OF THE FINDINGS:

Cerebral ischemia was induced by the middle cerebral artery occlusion or pretreatment of high fat diet and followed by middle cerebral artery occlusion, which caused impairment of neurobehavioral activities. It was confirmed by alteration in neurobehavioral parameters (Locomotor activities and grip strength tests), generation of free radicals or oxidative stress that was observed by elevation of (TBARS, GGT, acetylcholinestrase, nitrite levels) and reduction of antioxidant biomarkers (SOD, CAT, GSH) including Na^+,K^+-ATPase. There was also elevation of the inflammatory mediator (TNF- α), apoptotic factor (Caspase-3) and cerebral infarct size.

Guggulipid is an ethyl acetate extract of Guggul i.e. oleogum resin of Commiphora *wightii* (Arn.) Bhandari. E and Z guggulsterones were found in guggulipid. Phenolics, flavonoids and guggulsterones (E and Z) were found in guggul.

Locomotor activity and grip strength were improved in pretreatment of guggulipid, aspirin, their combination and atorvastatin groups after inducing cerebral ischemia as compared to MCAO and HFD + MCAO groups. The motor performance improved as evidenced by improvement in locomotor activity and grip strength tests may be a sign of reduction in ischemic injury in territory region.

GSH, catalase and SOD levels were elevated in pretreatment groups of guggulipid, atorvastatin and aspirin. Combination therapy of guggulipid and aspirin showed more significant improvement.

Elevation of these antioxidant enzymes levels were due to the antioxidant and neuroprotective properties of guggulipid which is responsible for reducing oxidative damage.

Na^+, K^+-ATPase levels were elevated in pretreatment groups of guggulipid, atorvastatin and aspirin. Combination therapy of guggulipid and aspirin also showed significant improvement.

This indicates that guggulipid protects the mitochondrial damage during ischemia and restores the ATP levels

TNF- α, Caspase- 3, Gamma glutamyl transferase, acetylcholinestrase and nitrite levels were significantly reduced in pretreated groups indicating the anti-inflammatory, anti-apoptotic, and neuroprotective activity of guggulipid. Due to reduction in acetylcholinestrase activity, the regional blood flow was increased.

Pretreatment of guggulipids, aspirin, their combination and atorvastatin significantly reduced the infarction areas as compared to MCAO and HFD with MCAO model in rat, indicating the neuroprotective properties of guggulipid.

On the basis of results from our study, it may be concluded that guggulipid (50mg/kg and 100 mg/kg) reduced the ischemia induced injury, improved the neurobehavioral deficits, and reduced oxidative stress, inflammatory marker and infarct size. Guggulipid dose (100 mg/kg) showed more improvement as compared with guggulipid dose 50 mg/kg and aspirin and atorvastatin individual dose. The results of the present study also showed more significant improvement when combined with aspirin in comparison to guggulipid alone.

With the additional anti-ischemic activity observed in this study, the previous reported actions of guggulipid such as anti-hyperlipedemic, anti-inflammatory, anti-platelet and anti-atherosclerotic effects may be useful in reversing the symptoms and may offer significant neuroprotection in

stroke. Our results are preliminary and more research is warranted to confirm the therapeutic role of guggulipid in ischemic stroke.

METHODOLOGY

Experimental Animals

Swiss strain albino Wistar male rats (250–300 g) were procured from the Central Animal House of Jamia Hamdard, New Delhi, India. All animals were kept in cages at a temperature of 22–30 ºC, natural 12 hour light/12 hour dark cycle and controlled humidity (55±5%) provided with standard pellet diet and tap water ad libitum. The protocol of research work was approved by Institutional Animal Ethics Committee Jamia Hamdard, New Delhi. Ethical norms were strictly followed in all the experimental procedures.

Drugs and Chemicals

(A). Guggulipid:

Guggulipid, an ethyl acetate extract of the resin of plant Commiphora *wightii* (Arn.) Bhandari or guggul was prepared. Guggul was purchased from Global Herb, Khari Bawli market, Old Delhi. The resin of plant Commiphora *wightii* (Arn.) Bhandari was identified by Dr. H. B. Singh at National Institute of Science Communication and Information. Resources (NISCAIR), New Delhi (Ref. No. NISCAIR/RHMD/Consult/2011-12/1081/281). Quality and purity of test drug were established according to Indian Pharmacopoeia, in Department of Pharmacognosy and Phytochemitry lab, Jamia Hamdard by estimating guggulsterones Z and E.

(B). Reference standards guggulsterone E and Z:

Reference standard E-isomer (purity 99.34%, w/w) and standard Z-isomer of guggulsterone (purity 99.07%, w/w) were procured from Sigma Aldrich, Shivaji Marg, New Delhi, India.

(C). High fat diet (HFD):

High fat diet was procured from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, Andhra Pradesh, India. HFD contains (g/kg): Casein-342g; Cystine-3g; Starch-172g; Sucrose-172; Cellulose-50g; G. N. oil- 25g Tallow-90g; Mineral oil-37g; Vitamin Mix-10g.

(D). Aspirin:

Aspirin was procured from USV limited, Govandi, Mumbai.

(E): Atorvastatin: It was procured from Dr. Reddy's laboratories LTD, Solan.Himachal Pradesh.

Diagnostic kits and Chemicals:

Diagnostic kits:

TNF- α	Krishgen Biosystem (India)
Caspase-3	Biovision (USA)
Gamma- glutamyl transferase (GGT)	Transasia Bio-medical LTD. Solan

Chemicals: All other chemicals used were of analytical grade. Double distilled water was used for all biochemical assays.

Plan of Work

The present study was carried out by following method as given below:

Identification and authentication of guggul:

The drug was identified and authenticated by Dr. H.B. Singh at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi as a oleo gum resin of Commiphora *wightii* on the basis of its organoleptic properties. Quality and purity of test drug was established according to Indian Pharmacopoeia, in Department of Pharmacognosy and Phytochemitry lab, Jamia Hamdard by estimating guggulsterones Z and E.

Preparation of guggulipid:

Guggulipid was prepared from 100 g air-dried resins; it was extracted with 500 ml of ethyl acetate for five days. The extract was filtered with Whatman no. 2 filter paper. The filtrate was collected and the solvent was evaporated under reduced pressure using vacuum evaporator. The dried extract was kept in a desiccator at a room temperature and was expressed in terms of dry weight (percentage yield 43.7%).

Characterization:

- **Determination of percentage yield:**

  The dried extract was kept in a desiccator at room temperature and expressed in terms of dry weight (% yield 43.7).

- **Determination of total phenolic content in guggul (Pourmorad et al., 2006):**

  Principle: Phenolic compounds are a class of antioxidant agent which acts as free radical terminators.

  Reagents: 10 % Folin Ciocalteu (F.C) reagent in distilled water, Na_2CO_3 (1 M) in distilled water and Standard (Gallic acid) 1mg/ml solution in methanol was prepared. Different dilutions of standard gallic acid (25 µg/ml to 300 µg /ml) were made in methanol.

Preparation of F.C. Reagent: The commercial F.C reagent was diluted (1:10) with distilled water on the day of use.

Preparation of aqueous Na_2CO_3: 1 M Na_2CO_3 was prepared by dissolving 106 g of Na_2CO_3 in 1000ml distilled water.

Samples preparation: Solution of Commiphora wightii (Arn.) Bhandari (10 mg/ml) were prepared in methanol followed by addition of 5ml F.C. reagent and 4 ml Na_2CO_3 solution. Absorbance was taken at 765 nm after 15 minutes.

Preparation of Standard: 0.5 ml of each std. dilutions was taken and added 5ml F.C. reagent and 4ml Na_2CO_3 solution. Absorbance was taken at 765 nm after 15 minutes.

Blank solution: Methanol (0.5 ml) and F.C. reagent (5ml) was taken. 4ml Na_2CO_3 solution was added to it.

- **Determination of total flavonoid content in guggul (Chang *et al.*, 2002):**

- 1 gm of sample of guggul was mixed with 3 ml of ethanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and it was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with UV/Visible spectrophotometer.

- The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg/ml ethanol.

- **Determination of physicochemical properties:**

These methods are taken from Ayurvedic pharmacopoeia of India (2007).

- **Ash value:**

Air dried guggul was weighed accurately and placed and ignited in crucible, gradually increasing the heat to 450°C until it is white, indicating the absence of carbon. Material was allowed to cool in desiccators and weighed.

Ash value can be determined by using formula:-

$$\text{Ash value} = \frac{(\text{Initial Weight} - \text{Final Weight}) \times 100}{\text{Final weight}}$$

- **Loss of drying:**

1.5 gram of guggul was dried in the oven at 105º C to constant weight and loss of drying was at 105ºC was determined.

- **Water soluble extractive value:**

Air dried amount (15 gm) of guggul was accurately weighed in a glass stoppered conical flask. 300 ml water was added to flask and weighed to obtain the total weight including the flask. After shaking well, the flask was allowed to stand for one hour. Reflux condenser was attached to flask and mixture was boiled for 6 hours. After that cooling and weighing were done. After filteration, extract was allowed to dry in oven. Content of extractive matter was calculated in mg per gm of air dried material.

$$\text{Extractive value} = \frac{(\text{Initial weight} - \text{final weight}) \times 100}{\text{Initial weight}}$$

- **Alcohol soluble extractive value:**

All the process was same as water soluble extractive value except that in place of water, alcohol was used in flask to make mixture.

- **Ethyl acetate soluble extractive value:**

All the process was same as water soluble extractive value except that in place of water, ethyl acetate was used in flask to make mixture.

- **Standardization of guggulipid:**

The extract was standardized with high-performance thin-layer chromatography (HPTLC) analysis by estimation of E- and Z-guggulsterones.

Estimation of Guggulsterones by HPTLC

The extract was standardized with high-performance thin-layer chromatography (HPTLC) analysis. The method developed by using stationary phase aluminum plates precoated with silica gel and mobile phase chloroform–methanol (9:1, v/v). The TLC plates were scanned using TLC Scanner 3 (Camag, Mumbai, India) at 259 nm (fluorescence/reflectance mode). Rf values of E & Z-gugguelsterones were 0.43±0.02 and 0.58±0.02 respectively. The linear regression analysis data for the calibration plots for E- and Z guggulsterones r^2= 0.9977±0.054 and 0.9975±0.068 respectively in the concentration range of 100–6000 ng/spot and the mean value of slope and intercept were 0.11±0.006 and 0.11±0.005, 14.26±0.56 and 10.92±0.76, respectively. Validation parameters summarized in the Table 4,2.1. Rf values found for E & Zguggulsterones in the herbal extract and drug samples were 0.43 and 0.58 along with others compounds. The guggulipid extract contained 4.1% of Z-E guggulsterone (2.5% of Z- guggulsterone and 1.6% of E-guggulsterone).

Validation parameters

Parameter	Data on guggulsterone	
	E	Z
Limit of quantitation	24 ng/spot	20 ng/spot
Limit of detection	12 ng/spot	10 ng/spot
Recovery (n=6)	100.16±1.13	100.69±1.45
Precision (R.S.D.%)		
Repeatability of application (n=7)	1.08	0.45
Repeatability of measurement (n=7)	0.99	0.32
Intra-day (n=6)	0.87	1.2
Inter-day (n=6)	1.29	1.68
Specificity	Specific	Specific
Robustness	Robustness	Robustness

Study I (MCAO MODEL): Evaluation of guggulipid in middle cerebral artery occlusion (MCAO) model of focal cerebral ischemia in rat.

Drug Administration:
Administration of pretreatment of oral doses (50 mg/kg and 100 mg/kg) of guggulipid, aspirin (100 mg/kg), their co-administration and atorvastatin (20 mg/kg) in different group of rats.

Weighed amount of guggulipid dose 1 (50 mg/kg), dose 2 (100 mg/kg), atorvastatin (20 mg/kg) and aspirin (100 mg/kg) were dissolved in normal saline using 0.5% carboxy methyl cellulose (CMC) as suspending agent. Calculated doses of drugs were given to rats per orally per day for 28 days. Normal control group and sham-operated animals received normal saline. Animals were divided into fourteen groups; each group consisted of eight rats, receiving different treatments for 28 days. The last dose of all drug treatment was administered three hours before inducing cerebral ischemia in rats.

Cerebral ischemia was induced for two hours followed by reperfusion for 22 hours. The rats were observed for behavioral parameters and then immediately sacrificed for infarct areas and estimation of oxidative stress parameters of whole brain.

Induction of cerebral ischemia by middle cerebral artery occlusion (MCAO) in rat (Longa et al., 1989)
Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in dorsal recumbency. A longitudinal incision of 1.5 cm in length was made in the midline of the ventral cervical skin. The right common carotid artery, ICA and ECA exposed and carefully isolated. A doccol monofilament (4.0 mm in length and 0.24 mm in diameter), inserted from the lumen of the ECA to that of the right ICA to occlude the origin of the right middle cerebral artery (MCA). The right MCA occluded for 2 hrs, and thereafter brain was allowed to be reperfused with blood by withdrawing of the monofilament. 24 h after reperfusion, behavioral studies were carried out. Immediately after, rat was decapitated, brain was removed for biochemical estimation.

Treatment schedule in MCAO model of rat.

Sr. no.	Group (n = 8)	Treatments
1.	Control	Normal saline 2 ml/kg, oral
2.	Sham	Normal saline 2 ml/kg, oral
3.	Guggulipid 1	50 mg/kg, oral
4.	Guggulipid 2	100 mg/kg oral
5.	Aspirin	100 mg/kg oral
6.	Guggulipid 1 + Aspirin	50 mg/kg oral + 100 mg/kg oral
7.	Guggulipid 2 + Aspirin	100 mg/kg oral + 100 mg/kg oral
8.	MCAO group	Normal saline 2 ml/kg, oral
9.	Guggulipid 1 + MCAO	50 mg/kg oral
10.	Guggulipid 2 + MCAO	100 mg/kg oral
11.	Aspirin + MCAO	100 mg/kg oral
12.	Atorvastatin + MCAO	20 mg/kg oral
13.	Guggulipid 1 + aspirin + MCAO	50 mg/kg oral + 100 mg/kg oral
14.	Guggulipid 2 + aspirin + MCAO	100 mg/kg oral + 100 mg/kg oral

Locomotor activity monitoring system (Morisset et al., 1999).

Locomotor activity was observed in open field arenas consisting of an acrylic box (40.6×40.6×40.6 cm^3) accommodated with two photo beam frames (16 beams/dimensions; 2.5 cm between beams; Coulbourn Instruments, Allentown, PA). The horizontal locomotor activity was recorded by the lower frame (2.5 cm above the arena floor) while the upper frame (15 cm above the floor) records rearing. The open field chamber was joined to a computer running software (TruScan 2.0 version, Coulbourn Instruments, Allentown, PA) that recorded beam breaks (100 ms sampling rate). Rats were kept for half an hour in home cage for habituation. Then they were placed in an open field chamber for half an hour prior to observe the locomotor activity (Danish et al., 2012; Morisset et al., 1999). Locomotion activity was recorded for 20 minutes during which horizontal locomotor activity was recorded of each rat. A total of 5 locomotor activity components were recorded for each groups as mentioned below:

- Horizontal activity (cm) - It is determined by measuring the total distance covered by a rat during the trial period.
- Total movements (#) - It is the observation of the total number of stereotypic movements exhibited by a rat during the fixed period.
- Movement time (s) - total time spent in exploring open field by a rat during the trial period is movement time.
- Rest time (s) - It is total time of rest by a rat during the trial period.

Grip strength test (Ali et al., 2004).

Grip strength meter was used for recording the grip strength of the animal (Ali et al., 2004). The animal's front paws were placed on the grid of grip strength meter and were moved

down until its front paws grasping the grid were released.The force achieved by the animal was then displayed on the screen and was recorded as kilogram unit.

Nitrite estimation (Aggarwal et al., 2010)

The accumulation of nitrite in the brain supernatant, an indicator of the production of nitric oxide (NO), was determined by a colorimetric assay with Griess reagent (0.1% N-(1-naphthyl) ethylene diamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid; Aggarwal et al., 2010). Equal volumes of Griess reagent and supernatant were mixed after that the mixture was incubated for 10 minutes at room temperature in the dark, and the absorbance at 540 nm was determined with Perkin Elmer lambda 20 spectrophotometer. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and expressed as percentage of control.

Catalase estimation (Clairborne, 1985)

Principle: In the Ultra Violet range H_2O_2 gives a continuous elevation in absorption with reducing wavelength. The breakdown of H_2O_2 can be followed directly by elevation in absorbance at 240 nm. The difference in absorbance (Δ A) per unit time is an estimation of catalase activity.

Reagents for the catalase estimation

Reagents	**Preparation of reagents**
Potassium dihydrogen phosphate (KH_2PO_4)	KH_2PO_4 was mixed in double distilled water and volume prepared up to 1000 ml with double distilled water
Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	8.90 g $Na_2HPO_4.2H_2O$ was putted in double distilled water and volume made up to 1000 ml
Potassium phosphate buffer (50 mM/l, pH 7.4)	It was prepared by mixing KH_2PO_4 solution and $Na_2HPO_4.2H_2O$ solution of 1:1.55
Hydrogen peroxide (30%)(19 mM/L)	187µl H_2O_2 **(**30%**)** was mixed in 100 ml phosphate buffer

Procedure:

The brain tissue was homogenized in 50 mM potassium phosphate buffer (pH 7.4) in ratio of 1:10 w/v. The homogenate was given for centrifugation at 10,000 rpm at 4 ºC in cooling centrifuge for 20 minutes. 50 µl supernatant was putted in cuvette containing 2.95 ml H_2O_2 (19

mM/L) solution prepared in potassium phosphate buffer. The disappearance of H_2O_2 was observed at 240 nm wavelength at 1 min interval for 3 minutes.

Calculation:

Catalase activity was determined by using the following equation and result expressed as nmole of H_2O_2 consumed/minute/mg protein.

$$\text{Catalase activity} = \frac{\Delta A/\text{minute} \times \text{total volume of assay}}{\text{Sample volume} \times \text{mg of protein}}$$

Gamma glutamyl transferase (GGT)

Gamma glutamyl transferase (GGT) is an enzyme abundantly present on the apical surface of endothelial cells and a reliable marker for the blood–brain barrier (BBB). It mediates transport of the γ-glutamyl moiety of glutathione and other γ-glutamyl donors from the apical surface (blood vessel lumen) to synthesize peptides within the endothelial cells inflammation and oxidative stress alter the activity of GGT. In this study, we measure the dynamic changes of GGT in cerebral microvessels after stroke by transient middle cerebral artery occlusion (tMCAO).

Reagent Compostion:

Active ingredients	**Contents of the test**
Tris buffer (pH 8.2 ± 0.1 at 20^0 C)	100 mmol/L
Glycylglysine	100 mmol/L
L-γ- glutamyl-3-carboxy-4-nitroanilide	2.9 mmol/L

Assay parameters:

Mode	**Kinetic**
Wavelength (nm)	405
Sample volume (µl)	50/100
Reagent volume (µl)	500/1000
Lag time (sec)	30
Kinetic interval (sec)	60
No. of reading	3
Kinetic factor	1156
Reaction temperature	37
Reaction direction	Increasing

Volumes requirement for assay: mixed and aspirated these.

Pipette in tubes	Volumes
Working reagent	1000 µl
Test	100 µl

Calculation:

The common formula for converting absorbance change into international unit (IU) of activity is:

$$\text{IU/L} = \frac{\Delta \text{ A/ min x T.V ml) x10}^3}{\text{S.V x Absorptivity x P}}$$

Where

T.V = Total reaction volume in µl

S.V = sample volume in µl

Absorptivity = millimolar absorptivity of L- γ- glutamyl -3 – carboxy- 4- nitroanilide 405 nm = 9.5.

P = cuvette lightpath (cm).

= 1 cm

Activity of GGT at 37^0 C (IU/L) = (ΔA_{405}/min) x Factor (1158)

Thiobarbituric acid reactive substances (TBARS) (Okhawa et al., 1979)

Lipid peroxidation is a type of free radical mediated phenomena. The prime products of such damage are a composite mixture of peroxides which disrupt to produce carbonyl compounds. The malondialdehyde (MDA) is like carbonyl compound, which makes a specific chromogenic adduct along two molecules of thiobarbituric acid to cast a pink color, the absorbance of which is determined at 540 nm. The colorimetric reflex of thiobarbituric acid (TBA) with MDA, a secondary creation of lipid peroxidation, is called as thiobarbituric acid reactive substance, and has been a well known accepted marker for lipid peroxidation.

Reagents for measurement of the lipid peroxidation

Reagents	**Preparation of reagents**
Potassium chloride (KCl) (0.15 M)	KCl (2.3 g) mixed in double distilled water (200 ml)
TBA solution (0.8%)	TBA (0.8 g) mixed in double distilled water (99 ml) and glacial acetic acid (1ml)
Trichloroacetic acid (TCA) solution (30 %)	TCA (30 g) added in double distilled water (100 ml)

Procedure:

According to that, 0.1 ml of homogenate, 1ml of trichloroacetic acid (10%) and 1ml of thiobarbituric acid (0.67%) were added to test tubes. All test tubes were covered with aluminum foil and placed in boiling water bath for 20 minutes. Then, all test tubes were placed in crushed ice bath and centrifuged at 6000 rpm for 10 minutes. The absorbance of all the supernatant was measured at 540 nm at room temperature against the appropriate blank. Blank consisted of 1 ml distilled water, 0.5 ml of TCA (30%) and 0.5 ml of TBA (0.8%).

Calculations:

The amount of MDA found in a sample was determined according to the following equation and the result was expressed as nmole MDA/mg of protein.

$$\text{nmole of MDA} = \frac{\text{Volume of assay} \times OD_{540}}{0.156}$$

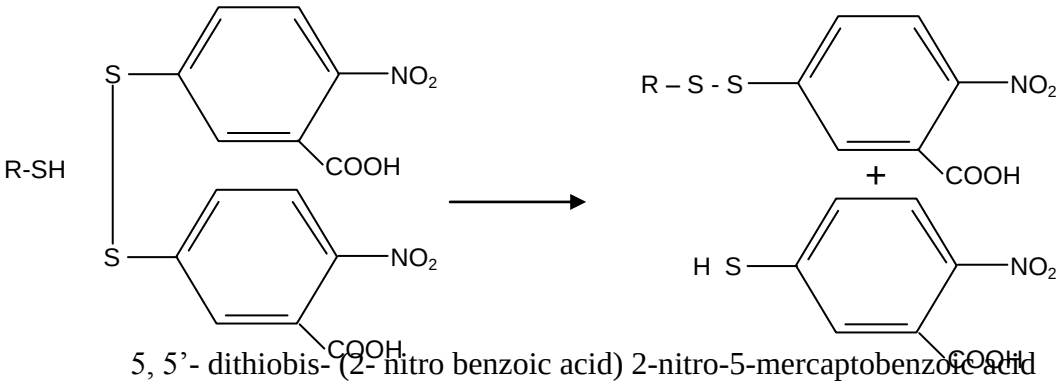
V = Final volume of test solution; OD (540) = Optical density at 540 nm

Reduced Glutathione (Sedlak and Lindsay, 1968)

Principle:

The glutathione estimation associates spectrophotometric analyses established on method previous described by Ellman. The Ellman reagent, 5, 5'-dithiobis-(2-nitrobenzoic acid (DTNB), is reduced with SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH.

Reaction involved in the conversion of DTNB



5, 5'- dithiobis- (2- nitro benzoic acid) 2-nitro-5-mercaptobenzoic acid

Reagents for the measurement of reduced glutathione.

Reagents	Preparation of reagents

Ethylenediaminetetraacetic acid (EDTA) (0.2 M)	22.3 g EDTA was dissolved in 300 ml warm double distilled water to prepare EDTA (0.2 M)
EDTA (0.02 M)	20 ml EDTA (0.2 M) diluted with use of 200 ml double distilled water
Tris buffer (0.4 M), pH 8.9	24.2 g Tris-buffer was dissolved in 100 ml double distilled water and 100 ml EDTA (0.2 M) incorporated to it and volume made up to 1000 ml with double distilled water. pH adjusted to 8.9 with 1 N hydrochloric acid (HCl)
DTNB (0.01 M)	99 mg DTNB was dissolved in 25 ml absolute methanol
TCA (50%)	50 g TCA was dissolved in 100 ml double distilled water

Procedure:

The equal amount of brain homogenate (w/v) and 10% trichloroacetic acid were mixed and centrifuged. A 2 ml of phosphate buffer (pH 7.4), 0.5 ml 5,5-dithiobisnitro benzoic acid (DTNB) and 0.4 ml of double-distilled water were added to 0.01 ml of supernatant. Then, the mixture was vortexed and their absorbance was recorded at 412 nm within15 minutes.

Standard:

The method was same to the test except that standard glutathione solution (50µg/ml) was taken in place of tissue homogenate.

Calculation:

The GSH in brain tissues was determined from the following equation and expressed as µg/ml of protein.

$$C_o = \frac{A_o}{A} \times C \times D.F.$$

C_o = concentration of GSH in Tissue (µg/mg wet tissue); A_o= O.D. of sample at 412 nm; A = O.D. of standard at 412 nm; C = concentration of standard GSH; and D.F. = dilution factor.

Superoxide dismutase estimation (Beauchamp & Fridovich, 1971)

Pyrogallol (1, 2, 3-benzenetriol) causes auto-oxidizes immediately in aqueous solution, greater the pH faster is auto-oxidation and several intermediate products are formed. Hence, the solution first turns yellow-brown with a spectrum showing between 400-425 nm. After few minutes the colour turns to green and finally after a few hours, a yellow colour forms. So auto-oxidation was studied essentially in the first step and the rate was taken from the linear increase in absorbance at 420 nm, which may seen for a number of minutes after an induction period of about 10 s. Superoxide anion radicals (O_2^-) tends to catalyze the auto-oxidation of pyrogallol. A simple and

rapid method for assay of SOD is followed, based on the property of the enzyme to decrease the auto-oxidation of pyrogallol.

$$2\ O_2^- + 2\ H^+ \longrightarrow O_2 + H_2O_2$$

Reagents for the measurement of superoxide dismutase activity

Reagents	Preparation of reagents
Tris HCl buffer (pH 8.5)	788 mg Tris HCl buffer and 186 mg EDTA mixed in 100 ml double distilled water and pH adjusted to 8.5 with 1 N NaOH.
Potassium phosphate buffer (50 mM/l, pH 7.4)	KH_2PO_4 solution and $Na_2HPO_4.2H_2O$ solution
Pyrogallol (24 mM)	15.1 mg pyrogallol was added in 5 ml HCl (10 mM)

Procedure:

Superoxide dismutase activity was measured by the method of Beauchamp & Fridovich [26]. The supernatant was assayed for SOD activity by following the inhibition of pyrogallol autoxidation. A 100 ml of cytosolic supernatant was added to Tris–HCl buffer (pH 8.5). The final volume of 3ml was adjusted with the same buffer. At least 25 ml of pyrogallol was added and changes in absorbance at 420 nm were recorded at one-minute interval for three minutes. The increase in absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD.

Calculation:

1 unit of SOD was defined as the amount of enzyme need to cause 50% reduction of pyrogallol auto-oxidation per 3 ml of assay mixture and followed by the formula:

$$\text{Unit of SOD / ml of sample} = \frac{(A\text{-}B) \times 100}{A \times 50}$$

A = Change in absorbance per minute of standard; B = Change in absorbance per minute of test sample.

Acetylcholine esterase estimation (Ellman et al, 1961)

Principle:

The rate of creation of thiocholine from acetylthiocholine in the presence of tissue cholinesterase is estimated by first treating with DTNB and then determining the optical density of the yellow

colored compound developed during the reaction of at 412 nm, every minute for a period of three minute. "AChE activity" was appropriated as µmol acetylthiocholine iodide hydrolyzed/minute/mg protein.

Reagents for AChE estimation

Reagents	Preparation of reagents
Sodium Phosphate buffer (0.1M, pH 8.0)	689 mg monosodium dihydrogen phosphate and 709 mg disodium monohydrogen phosphate were added to dissolve in 80 ml double distilled water and the pH was adjusted to 8.0 with NaOH and final volume made up to 100 ml
5'5 bis-dithionitrobenzoic acid (DTNB) (10 mM)	39.6 mg DTNB and 15 mg anhydrous sodium carbonate were added in 10 ml of 0.1M phosphate buffer (pH 8.0)
Acetylthiocholine iodide (30 mM)	86.76 mg acetylthiocholine iodide was added in 10 ml 0.1 M phosphate buffer (pH 8.0)
Alkaline sodium carbonate solution	2 mg sodium carbonate was solubilized in 100 ml 0.1 N NaOH
Cupric sulphate solution and sodium potassium tartarate solution	500 mg cupric sulphate was solubilized in 100 ml distilled water to get a solution of 0.5%, and 1gm sodium potassium tartarate added to it
Alkaline solution	Prepared by mixing 50 ml alkaline sodium carbonate solution and 1 ml cupric solution and sodium potassium tartarate solution
Folin's ciocalteau phenol reagent	The commercial reagent was freshly prepared by diluting with 2 volumes of distilled water

Procedure:

Rat brain was decapitated and the whole brain AchE was estimated according to the method of Ellman et al. (1961). Brain tissue of known weight was homogenized in 0.32M sucrose solution and 10% of homogenate was centrifuged at 3000 rpm for 15 minutes followed by centrifugation at 10 000 rpm for 10 minutes, at a constant temperature of 4^0 C. A 9 ml of sucrose solution was mixed with 1ml of supernatant. Test samples were prepared by mixing 2.7 ml of phosphate buffer, 0.1 ml of DTNB and 0.1 ml of postmitochondrial supernatant (PMS). The reaction mixture was pre-incubated for five minutes, and 0.1 ml of acetylthiocholine iodide was added to the mixture to initiate the reaction and immediately absorbance was taken at 412 nm for three minutes every one-minute interval. Blank sample was made in the similar manner except PMS was not added. Protein was determined according to the method of Lowry et al. (1951).

Estimation of acetylcholinesterase activity

Reagents	Blank (ml)	Test (ml)	
Phosphate buffer	2.8	2.7	
DTNB	0.1	0.1	The mixture was incubated for 5 min
PMS (1%)	0.0	0.1	
Acetylthiocholine iodide	0.1	0.1	Added in above incubated mixture

Calculation:

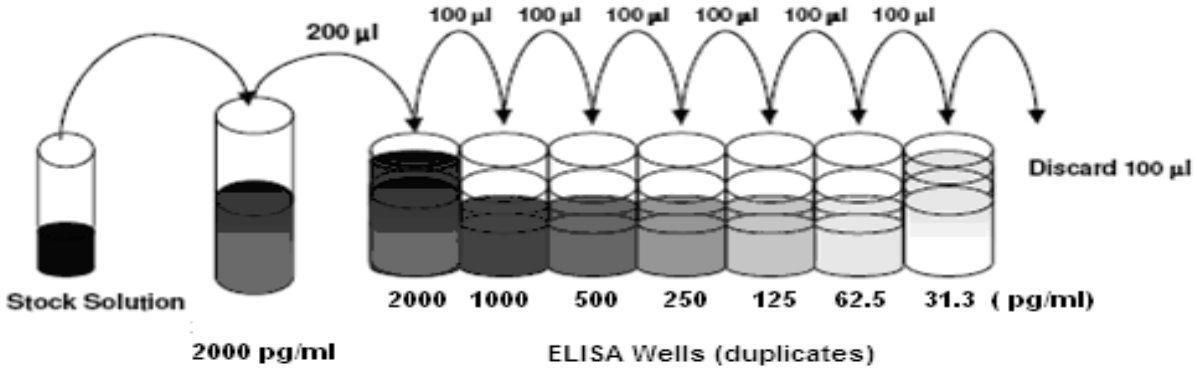
$$R = \frac{\Delta A \times \text{volume of assay (3 ml)}}{E\ (13.600) \times \text{mg of protein}}$$

R= rate of enzyme activity in 'n' moles of acetylcholine iodide hydrolyzed/minute/mg protein; Δ A = change in absorbance/minute, E = extinction co-efficient (13.600/M/cm).

TNF-estimation (Krishgen biosystem)

Assay Procedure:

1. All the reagents were taken to room temperature prior to use.
2. 100µl/well of Standards added to sample plate. Six two fold serial dilutions of the 2000pg/ml top standard were performed, either within the plate or in separate tube. Hence the TNF- alpha standard concentrations are 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, and 31.3pg/ml. Diluent taken as zero standards. Plate was sealed and incubated for 2 hours at Room Temperature (18-25°C).



3. Plate was aspirated and washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper. Any liquid from outside the bottom wiped.

4. 100µl of diluted **Detection Antibody** solution was added to each well. Plate was sealed and incubated for 2 hours at Room Temperature (18-25°C).

5. Plate was washed with **Wash Buffer (1X)** 4 times as in step 3.

6. Each well was added with 100µl of diluted **Streptavidin-HRP** solution. Plate was sealed and incubated for 30 minutes at Room Temperature (18-25°C).

7. Plate was washed 4 times with **Wash Buffer (1X)** as in step 3.

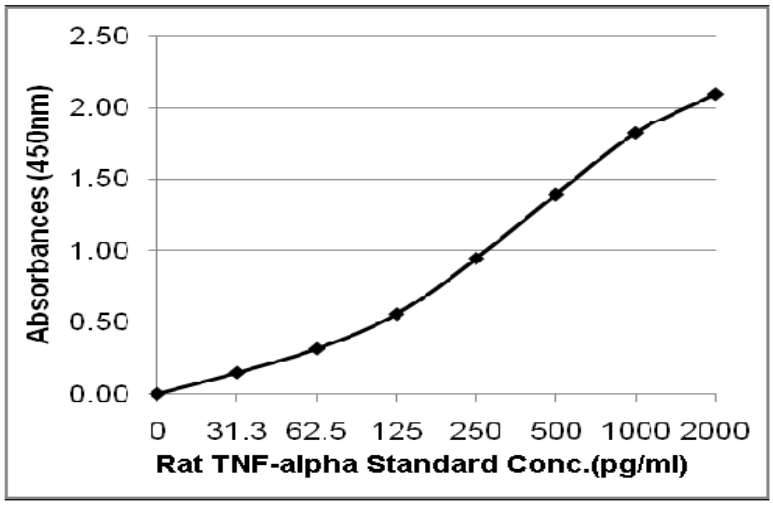
8. 100µl of **TMB Substrate** solution was added and incubated in the dark for 15 minutes. Positive wells turned bluish in color.

9. Reaction was stopped by adding 100µl of **Stop Solution** to each well. Positive wells turned from blue to yellow.

10. Absorbance was observed at 450 nm within 30 minutes of stopping reaction.

Calculation of Results:

Mean absorbance for each set of duplicate or triplicate standards and samples was dtermined. Mean absorbance of the zero standards (background) from each well were subtracted. Standard curve on Lin-Lin graph paper, with TNF- alpha level on the x-axis and absorbance on the y-axis ploted. The best fit straight line through the standard was drawed. points. To determining the unknown TNF- alpha levels, unknowns mean absorbance value on the y-axis were drawed a horizontal line to the standard curve. At the point of intersection, vertical line drawed to the x-axis and the TNF- alpha levels were determined.



Rat TNF- alpha standard concentration

Caspase-3 estimation (Ray biosystem)

Components	**K106-100 (100 assays)**
Cell Lysis Buffer	100 ml
2X Reaction Buffer	4 × 2 ml
DEVD-*p*NA (4mM)	0.5 ml
DTT (1M)	0.4 ml
Dilution Buffer	100 ml

Caspase-3 Assay Protocol by kit:

Assay Procedure

I. Cells in 50 µl of chilled cell lysis buffer were resuspended and incubated for 10 minutes.
II. Centrifugation of lysis buffer solution was done for 1 minute (10,000 x g).
III. Supernatant was putted to tube and tranfersed on ice for instant assay or kept at -80°C for further use.
IV. 50 µl of 2X Reaction Buffer (10 mM DTT) was putted to each sample.
V. 5µl from the 4 mM DEVD-*p*NA substrate (200 µM) was putted and allowed for incubation at 37°C for 1-2 hour.
VI. Samples were observed at 405-nm in a microliter plate reader

Infarct size estimation

Rats were sacrificed and their brains were removed after 24 hours of middle cerebral artery occlusion. The brains were sectioned into six slices each with 2mm thickness, immersed in 2% triphenyl tetrazolium chloride (TTC) dye for 30 minutes at a 37^0C. The brain tissue was differentiated according to white-colored infarct area and red-purple noninfarct area. The slices were placed on a glass plate in a row and the images were captured. Image J Software (NIH, Bethesda, MD) was used for the measurement of infarction (white area) and the total area of the section (red-purple). The percentage of the area of infarction was calculated as [(white or yellow area/total area) ×100] (Saraf et al., 2010).

Study II (High Fat Diet MODEL): Evaluation of guggulipid on high fat diet (HFD) and high fat diet induced changes on the MCAO model of focal cerebral ischemia in rat for its neuroprotective effects.

Guggulipid dose 1 (50 mg/kg), dose 2 (100 mg/kg), atorvastatin (20 mg/kg) and aspirin (100 mg/kg) were dissolved in normal saline using 0.5% carboxy methyl cellulose (CMC) as suspending agent. Calculated doses of drugs were given to rats per orally per day for 28 days.

Normal control group animals received normal control diet. High fat diet group and all groups except control of rats were fed high fat diet. Animals were divided into nine groups; each group consisted of eight rats, receiving different treatments for 28 days. The last dose of all drug

treatment was administered three hours before inducing cerebral ischemia in rats. Cerebral ischemia was induced for two hours followed by reperfusion for 22 hours. The rats were observed for same behavioral parameters and then immediately sacrificed for infarct areas and estimation of biochemical parameters of whole brain as done in model I.

Experimental groups and treatments plan in HFD fed MCAO model in rats

Sr. No.	Group (n = 8)	Treatments (28 days)
1.	Control	Standard diet with normal saline 2 ml/kg, oral
2.	HFD per se	High fat diet with normal saline 2 ml/kg, oral
3.	HFD + MCAO	High fat diet with normal saline 2 ml/kg, oral
4.	HFD + GL1 (50 mg/kg) + MCAO	High fat diet with guggulipid dose 50 mg/kg.
5.	HFD + GL2 + MCAO	High fat diet with guggulipid dose 100 mg/kg.
6.	HFD + ASP + MCAO	High fat diet with aspirin dose 100 mg/kg.
7.	HFD + ATORVA + MCAO	High fat diet with atorvastatin dose 20 mg/kg.
8.	HFD + ASP + GL1 + MCAO	High fat diet with guggulipid (50 mg/kg) + aspirin (100 mg/kg).
9.	HFD + ASP + GL2 + MCAO	High fat diet with guggulipid (100 mg/kg) + aspirin (100 mg/kg).

Statistical Analysis:

The data were expressed as mean ±SEM (standard error of mean) and results were analyzed with the help of Analysis of Variance (ANOVA) followed by Dunnet's t-test for multiple comparison tests. The statistical significance level was set at $p< 0.01$ %. The statistical analysis was carried out using software graph-pad prism 3.0 (San Diego, California, USA).

Results and Discussion

Identification and authentication of guggulipid

The guggul (oleogum resin of *Commiphora wightii*) was authenticated by Dr. H. B. Singh at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, on the basis of its organoleptic properties. Further quality, purity and authenticity of test drug were confirmed and established according to Indian Pharmacopoeia, in the Department of Pharmacognosy and Phytochemitry lab, Jamia Hamdard by estimating the guggulsterones (Z + E) using HPTLC.

Guggulipid is an ethyl acetate extract of oleo gum resin of Commiphora wightii (Arn.) Bhandari, which is identical to commercialized Gugulip® in its percentage of main active

constituent i.e. E+Z gugulsterone. Indian Pharmacopoeia quality parameters tests have been followed. According to Indian Pharmacopoeia gugulipid contains not less than 4.0 per cent and not more than 6.0 per cent of gugulsterones (Z and E). 4.1% of (E+Z) gugulsterones were found in our test drug.

Physicochemical properties of guggul

Physical description

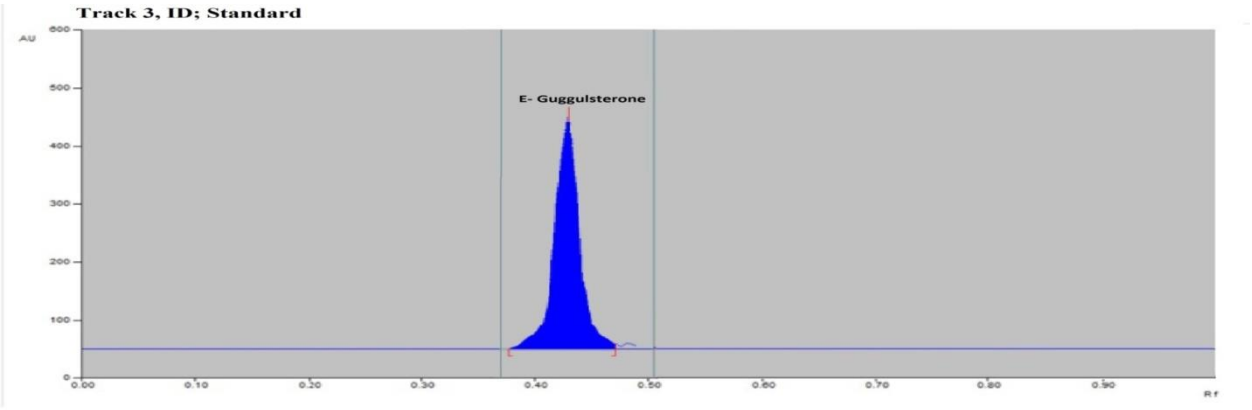
It is oleoresin of the plant *Commiphora wightii* (Arn.) Bhandari. The oleoresin is yellowish or pale yellow, aromatic fluid flows out that turns into an "agglomerate of tears or stalactic pieces" that are reddish brown, golden brown, or dull green.

Preliminary Phytochemical Screening of Guggul showed to contain, lipids steroid, phenolic and flavonoid components.

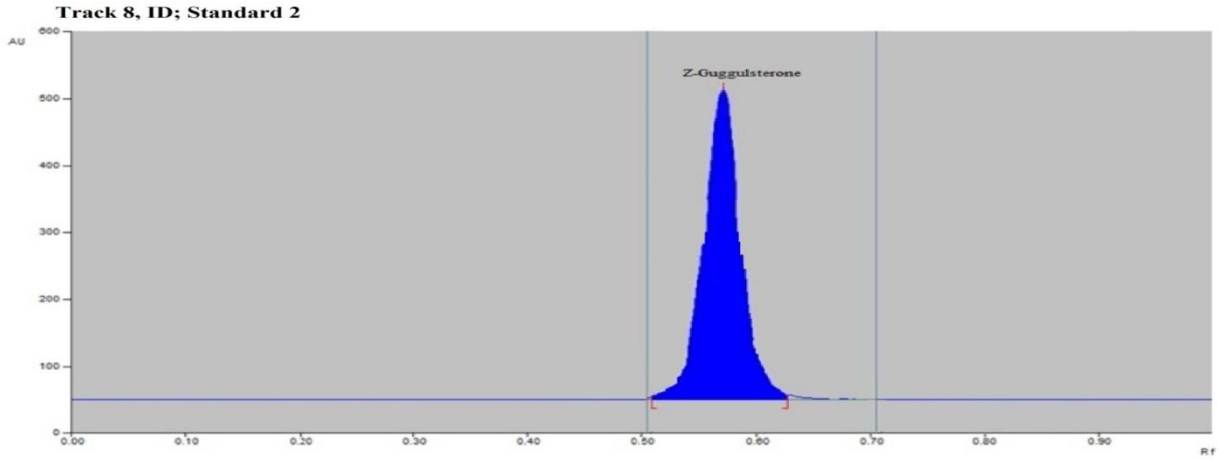
HPTLC fingerprinting of guggulipid extract

The extract was standardized with high-performance thin-layer chromatography (HPTLC) analysis. A stock solution (1 mg/ml) guggulipid extract was prepared in methanol and analyzed by HPTLC (Camag). Guggulsterone, which is the principal constituent of guggulipid, was used as standard, and the solution (0.1 mg/ml) was prepared in methanol. Calibration curve was obtained for 100–6000 ng/spot concentrations of guggulsterone. The developing solvent system used was chloroform: methanol (9:1). The TLC plates were scanned using TLC Scanner 3 (Camag, Mumbai, India) at 259 nm (fluorescence/reflectance mode). The guggulipid extract contained 4.1% of Z-E guggulsterone (2.5% of Z-guggulsterone and 1.6% of E-guggulsterone).

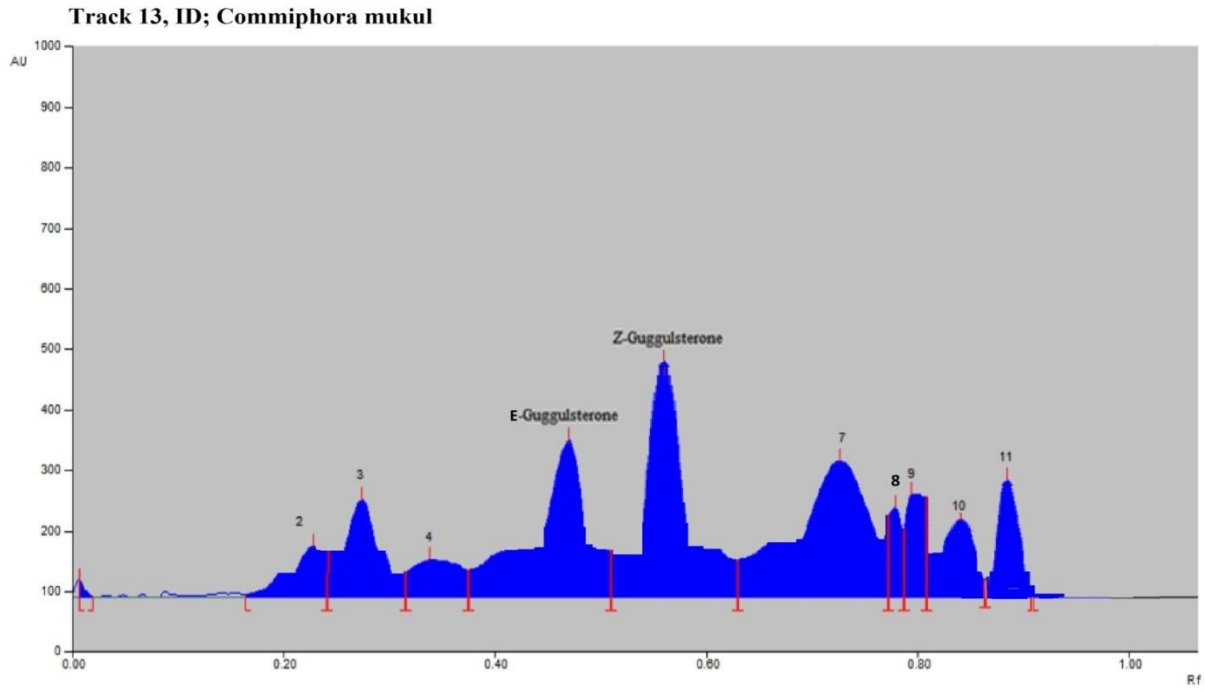
HPTLC fingerprinting of E- guggulsterone



HPTLC fingerprinting of z- guggulsterone



HPTLC fingerprinting of guggulipid extract



Total phenolic content of guggul

Sr. No.	Absorbance (765 nm)	Total phenolic contents (mg/g)	Mean ± SEM
1.	1.3	12.08	12.69 ± 0.46
2.	1.5	13.6	
3.	1.35	12.4	

Total flavonoid content ethyl acetate extract of guggul

Sr. No.	Absorbance (415 nm)	Total flavonoid contents (mg/g)	Mean ± SEM
1.	0.516	5.86	5.74 ± 0.086
2.	0.474	5.57	
3.	0.509	5.8	

Preliminary phytochemical screening of guggul

Tests	Name of test	Oleo gum resin of guggul
Test for Steroids	Libermann Burchardt test	+++
	Salwaski Test	+++
Test for Flavonoid	Aqueous sodium hydroxide test	+
	Magnesium hydroxide tesy	+
Test for phenolics	Ferric chloride test	++
	Bromine water test	+

Preliminary phytochemical properties ethyl acetate extract of guggul

Sr. no.	Tests	Value (% age).
1.	Percentage yield	43.7
2.	Ash value of extract	4.9
3.	Loss of drying was at 105ºC	4.5
4.	Water soluble extractive value	51
5.	Ethanol soluble extractive value	43
6.	Ethyl acetate soluble extractive value	41

Srudy 1: **Evaluation of guggulipid extract in middle cerebral artery occlusion (MCAO) model of focal cerebral ischemia in rat.**

Cerebral ischemia causes significant impairment of motor performance test in rats. (Akhtar et al., 2012, 2014; Chaudhary et al., 2003). It was evident by the decreased in performance of grip strength and locomotor activity like move time, horizontal activity, mean velocity and total movement with increase in rest time. Neuronal damage in the territory of middle cerebral artery may be the possible reason of this deficit. There was a significant improvement observed in motor performance of pre-treatment of guggulipid, aspirin, atorvastatin, and guggulipid with aspirin combination group when compared with MCAO group. These observations indicated that the improvement in motor performance may be due to the reduction in ischemic injury in

territory region. There was more significant improvement in motor performance of the rats observed in pre-treated combination of guggulipid and aspirin followed by MCAO.

Guggulipid decreased the lipid peroxidation induced by MCAO which may be due to restoration of altered antioxidant, indicating its antioxidant property.

Various studies supported that there is production of free radicals in reperfusion induced cerebral ischemia in which peroxide and hydroxyl radicals are potent lipid peroxidation inducing agent. Free radical production or reactive oxygen species can cause cellular damage or neuronal death, because oxidation of cellular components like lipids, protein and DNA and alteration of signaling pathways that finally promote the damage of cells (Bora & Sharma, 2010).

Middle cerebral artery occlusion significantly increased the level of TBARS, which is the marker of lipid peroxidation when compared with the control and sham group. In postischemic condition, elevated levels of TBARS suggested that free radicals produced during reperfusion could be responsible for the neuronal injury (Pratap et al., 2010; Gupta et al., 2002). A significant reduction in TBARS levels was observed in guggulipid, aspirin, atorvastatin, and guggulipid with aspirin combination pre-treated group showing the antioxidant properties of guggulipid responsible for reducing oxidative damage. The combination of guggulipid and aspirin also showed a significant reduction in levels of TBARS, which confirmed the neuroprotective and/or anti-oxidant properties of both the drugs.

Glutathione (GSH), an endogenous antioxidant, which reacts with free radicals and protects free radical-induced ischemic damage (Kutsuna et al., 2010). In this study, GSH levels were significantly reduced in middle cerebral artery occluded rats, which could be due to the consumption of GSH in scavenging the rapidly generated reactive oxidative species, caused by acute ischemic stroke. It was observed that guggulipid. atorvastatin, and guggulipid with aspirin combination enhanced the GSH levels after middle cerebral occlusion, indicating that the elevation of GSH levels was due to the antioxidant effects of both guggulipid and aspirin. Free radical production is elevated after cerebral ischemia and particularly reperfusion injury disrupting the endogenous antioxidant systems. SOD and catalase that are helpful enzymes in reduction of oxidative stress were decreased in MCAO rats. Guggulipid. atorvastatin, and guggulipid with aspirin combination group elevated these antioxidant enzyme activities.

Several studies shown that high levels of NO generation were partially responsible for exacerbating the neuronal damage induced by MCAO by intraluminal filament (Dohare et al., 2008; Niranjan et al., 2010). It this study guggulipid. atorvastatin, and guggulipid with aspirin combination group significantly reduced the stimulated NO release followed by MCAO.

Acetylcholine esterase is the metabolizing enzyme of acetylcholine and its inhibition is important for increasing cholinergic neurotransmission and regional cerebral blood flow. Therefore, cholinesterase inhibition plays an important role in regional cerebral blood flow (Lojkowska et al., 2003). In this study, AchE enzyme activity was significantly increased in MCAO rats in comparison with control and sham group while guggulipid. atorvastatin, and guggulipid with aspirin combination pre-treatments showed significant inhibitory effect. These results indicate that chronic treatment of guggulipid, aspirin and their combination may increase the cholinergic activity followed by MCAO and guggulipid has anti-AchE activity, which can be responsible for increasing the regional cerebral blood flow and memory enhancement. Our observation confirms earlier reported work (Saxena et al. 2007).

It was found from previous study that there is significant correlation between gammaglutamyl transferase (GGT) and stroke (both hemorrhagic and ischemic types). According to the coronary artery risk development in young adults investigated 5115 individuals aged between 17 and 35

years. CARDIA study, it was observed that GGT is a sensitive initiative stage predictor. Elevated GGT levels predict functional impairment in adults after ischemic stroke.of oxidative stress (Lee et al., 2003; Ambrosio et al., 2013). Oxidative stress and inflammation can account for the role of GGT in the progression of cerebrovascular disease. In this present study, GGT enzyme activity was significantly increased in MCAO rats in comparison with control and sham group while guggulipid. atorvastatin, and guggulipid with aspirin combination pre-treatments significantly had inhibitory effect. Guggulipid showed inhibition of GGT level in brain which showed its anti-inflammatory and neuroprotective effect.

In the present study Sod-Pot ATPase (Na^+,K^+-ATPase) levels were significantly reduced in MCAO model. Reduction of Na^+,K^+-ATPase could be a result of mitochondrial damage during ischemia that prevents restoration of ATP levels. Na^+,K^+-ATPase levels were elevated in pretreatment group of guggulipid, atorvastatin and aspirin. Combination therapy of guggulipid and aspirin also showed significant improvement.

There is highly accumulation of inflammatory cells in neuronal inflammation produced due to reperfusion induced damaged areas of neuronal tissue. Tumor necrosis factor –α (TNF- α) an inflammatory cytokine produced by endothelial cells, astrocytes, macrophages and neuronal cells promote the inflammatory reactions in the brain. In this study pretreatment of guggulipid, atorvastatin and aspirin showed significant reduction in the inflammatory marker like TNF- α. There was a more significant improvement observed in combination group of guggulipid and aspirin. It was also suggested from various studies that guggulipid showed anti-inflammatory property in neuronal cells (Niranjan et al., 2010; Gupta et al., 2002).

Guggulipid was reported to possess E- and Z-guggulsterones, including C21, C27 sterols and other esters. E- and Z-guggulsterones are the main active constituents responsible for potent antioxidant, free radical scavenging, anti-inflammatory and anti-AchE activities (Deng et al., 2007: Urizar et al., 2003). A prominent infarction was seen in MCAO rat by TTC dye staining. The infarction area was significantly reduced as compared with MCAO rats when guggulipid, and aspirin alone and their combination were used. Administration of guggul (500 mg/kg) once daily for five days was reported to reduce the infarct area and improved the sensory and motor deficits in rats (Adam et al., 2002). Thus, the results of this study showed that chronic pre-treatment of guggulipid may be beneficial in preventing the symptoms of cerebral ischemia.

Study II. Evaluation of pretreatment of guggulipid, aspirin, atorvastatin and their co-administration on locomotor activity parameter in high fat diet fed middle cerebral artery occluded rats.

It has been reported that there is a significant impairment of motor performance in middle cerebral occluded rats and high fat diet rat (Ahmad et al., 2014; Ojha et al., 2008; Sarikaya et al., 2011). The possible reason of this dysfunction may be the neuronal damage in the territory of middle cerebral artery. The significant improvement was observed in motor performance of pre-treatment group of guggulipid, atorvastatin, and aspirin when compared with HFD and HFD + MCAO group. The improvement in motor performance may be a sign of reduction in ischemic injury in territory region. Pretreatment of guggulipid and aspirin showed more significant improvement in motor performance of the rats followed by MCAO. Various studies reported the production of free radical production or reactive oxygen species (ROS) in reperfusion induced cerebral ischemia and high fat diet induced obesity which can cause neuronal damage due to oxidation of cellular components like proteins, lipids alteration of signaling pathways that leads

to damage of neuronal cells (Akhtar et al., 2014). High fat diet and middle cerebral artery occlusion significantly enhanced the TBARs levels, which is marker of lipid peroxidation (Wu et al., 2010). Various studies suggested that elevated TBARs levels could be responsible for oxidative stress induced neuronal injury (Akhtar et al., 2014: Gupta et al., 2002; Sinha et al., 2001). In our study TBARs levels were significantly reduced in pretreated groups showing the antioxidant properties which are responsible for reducing oxidative damage.

Endogenous antioxidants like GSH, SOD and catalase act as detoxifying agents to detoxify the overproduction of free radicals. Physiologically SOD catalyzes the conversion of superoxide anions to hydrogen peroxide and molecular oxygen. Catalase and GSH are involved in scavenging of hydrogen peroxide (Li et al., 2013). In the present study GSH, catalase and SOD levels were significantly reduced in HFD and HFD + MCAO group which could be due to detoxifying the rapidly generated reactive oxygen species caused by high fat diet and acute ischemic reperfusion induced brain injury. Our study also observed a rise in GSH, catalase and SOD levels in pretreatment groups of guggulipid, atorvastatin and aspirin. In combination group of guggulipid and aspirin, GSH, catalase and SOD levels were also more significantly increased as compared with HFD and HFD + MCAO group, indicating that the elevation of these antioxidant enzymes levels were due to the antioxidant properties of pretreated drugs. Our observations are supported with other earlier reported observations (Akhtar et al., 2014).

There was an elevation of nitric oxide (NO) level found in HFD and HFD + MCAO group of rats. Pretreated drugs significantly reduced the potentiated NO release followed by high fat diet and middle cerebral artery occlusion. It was demonstrated from various studies that high levels of NO production participated for exacerbation of neuronal damage (Niranjan et al., 2010; Dohare et al., 2008). Hence the guggulipid pretreatment showed the neuroprotective property.

Increased GGT levels can play role in the evolution and instability of atherosclerotic plaques in different vascular areas. In previous study it was found that in acute ischemic stroke (AIS) patient GGT level was higher as compared with control group. Apprehension of relatively higher levels of GGT in AIS patients with increased LDL-cholesterol, and triglyceride levels indicates the presence of a positive correlation between GGT levels, oxidative stress, and inflammation.

There was an elevation of GGT level found in HFD and HFD + MCAO group of rats as compared with control group. Pretreated drugs significantly reduced the GGT level followed by high fat diet and middle cerebral artery occlusion. Hence the guggulipid pretreatment showed the neuroprotective property.

Na^+, K^+-ATPase levels were more significantly reduced in HFD + MCAO model. Reduction of Na^+, K^+-ATPase could be a result of mitochondrial damage during ischemia that prevents restoration of ATP levels (Yang et al., 1992). Na^+, K^+-ATPase levels were increased in pretreatment group of guggulipid, atorvastatin and aspirin. Combination therapy of guggulipid and aspirin also showed more significant improvement. This indicates that guggulipid protects the mitochondrial damage during ischemia and restores the ATP levels.

According to previous study, cerebral ischemia provokes two general pathways of apoptosis: the intrinsic pathway, arising from mitochondrial release of cytochrome *c* and accompliced stimulation of caspase-3; and the extrinsic pathway, arising from the activation of cell surface death receptors, promoting in the stimulation of caspase-8. Hence the simplistic concept that ischemic stroke-induced apoptosis happens predominantly in neurons and is caspase-dependent (Brad et al, 2009).

In this study elevation in Caspase-3 level was found in HFD and HFD + MCAO group of rats as compared with control group. Pretreated drugs like guggulipid, aspirin and atorvastatin

significantly reduced the Caspase-3 level followed by high fat diet and middle cerebral artery occlusion. Hence the guggulipid pretreatment showed the antiapoptotic property.

It was known from various studies that ischemic stroke is evoked by inflammatory reaction (Claiborne et al., 1985; Sinha et al., 2001). Brain inflammation produced during reperfusion is due to the accumulation of inflammatory cells and microvascular dysfunction in damaged areas of neuronal tissue (Ding et al., 2006).

According to previous study it was found that HFD cause neuroinflammation which is also responsible for the neuronal injury and or neuronal cells death (Pratibha et al., 2011). Tumor necrosis factor –α inflammatory cytokine produced by endothelial cells, astrocytes, macrophages and neuronal cells promote the inflammatory reactions in the brain. In the present study pretreatment of guggulipid, atorvastatin and aspirin showed significant reduction in the inflammatory marker like TNF- α. There was a more significant improvement observed in combination group of guggulipid and aspirin. It suggests anti-inflammatory property of guggulipid. Prominent infarction areas were seen in brain sections of HFD and HFD + MCAO groups by TTC dye staining. The infarction areas were significantly reduced in pretreatment groups of guggulipid, atorvastatin, aspirin as compared with HFD and HFD + MCAO groups of rats. In combination group of guggulipid infarctions were less as compared with individual drug of guggulipid, aspirin and atorvastatin. Therefore, the results of the present study demonstrated that chronic pre-treatment of guggulipid may be helpful in reducing the symptoms of cerebral ischemia in rats.

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8. CONTRIBUTION TO THE SOCIETY

The Ayurveda describes the use of herbal medicines for the treatment of various diseases. Nowadays herbal therapy is appreciated by many research workers. Herbal drugs have relatively more therapeutic window, lower side effects and more cost effective. Hence they have a lot of recognition in the modern era and can be potential contenders for prophylactic treatment of stroke. *Commiphora wightii* (Arn.) Bhandari commonly known as guggul (guggulipid) has been used for thousands of years in the treatment of arthritis, inflammation, obesity, cardiac protection, anti-ulcer, anti-epileptic and disorders of lipid metabolism. Preclinical studies support that gugulipid inhibit tissue factor and arterial thrombosis, delaying progression of atherosclerosis, impaired plasminogen activator inhibitor-1 as well as vascular cell adhesion molecule-1 protein, anti-hypercholesterolemic (Farnesoid X receptor antagonist), anti-inflammatory (Inhibit TNF-α expression, which is elevated in stroke) anti-oxidant properties and inhibits the platelet aggregation.

Clinical studies also support that guggulipid showed hypolipidemic effect (Farnesoid X receptor antagonist), anti-oxidant effect, anti-inflammatory effect due to inhibition of activation of NF-kappa B (responsible for ischemia induced neuronal injury), and cardioprotective effect in ischemic patients. But no work has ever been carried out to evaluate the effects of guggulipid on reperfusion induced cerebral injury. Middle cerebral artery occlusion (MCAO) followed by reperfusion is a model of focal ischemia in rats, which resembles that of stroke in human and is widely used for evaluating the therapeutic intervention. The present study demonstrated that guggulipid reduced the ischemia induced injury, ameliorated the neurobehavioral deficits, and reduced oxidative stress, inflammatory marker and infarct size in animals. It reported that pretreatment of guggulipid may be useful in reversing the symptoms and may offer significant neuroprotection in cerebral ischemia in animal models of stroke.

The work has already been published in reputed international journals.

9. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT:

One Ph.D student was enrolled and awarded the Ph.D degree.

10. NO. OF PUBLICATIONS OUT OF THE PROJECT (PLEASE ATTACH RE-PRINTS):

Two research papers were published.

1. Neuroprotective effects of guggulipid alone and in combination with aspirin on middle cerebral artery occlusion model of focal cerebral ischemia in rats. Ahmad MA, Najmi AK, Mujeeb M, **Akhtar M.** Toxicol Mech Methods 2014, 24(6):438-447. DOI: 10.3109/15376516.2014.939320 **(International, Impact Factor=1.54) ISSN:** 1537-6516. **(Paper Published).**

2. Protective effect of guggulipid in high fat diet and middle cerebral artery occlusion (MCAO) induced ischemic cerebral injury in rats. Ahmad MA, Najmi AK, Mujeeb M, **Akhtar M.** Drug Res (Stuttg) 2016; 66:407-414**.** (**International, Impact Factor =0.8) ISSN***:*** 2194-9379**(Paper Published).**

JAMIA HAMDARD
(Hamdard University)

Hamdard Nagar, New Delhi - 110062

(Declared as Deemed-to-be University under Section 3 of the UGC Act, 1956 vide Notification No. F.9-18/85-U.3 dated 10.5.1989 of the Government of India)

No. JH/Exam/N-Ph.D-069/16

December 5, 2016

Notification for the Award of Ph.D. Degree

It is hereby notified that Jamia Hamdard has accepted the thesis of under mentioned candidate for the degree of Doctor of Philosophy in the subject and Faculty mentioned below and the candidate has been declared eligible for the award of the degree of Doctor of Philosophy of this University.

Name	: Mr. Md. Afroz Ahmad
Father's name	: Mr. Md. Abdul Ghaffar
Enrolment No.	: 2004-305-026
Subject	: Pharmacology
Faculty	: Pharmacy
Title of the Thesis	: "Study of guggulipid in animal model of focal cerebral ischemia"
Name of the Supervisor	: Dr. Mohd. Akhtar
Name of the Co-supervisor	: Dr. Mohd. Mujeeb
Date of Viva-voce Examination	: November 25, 2016

Ph.D. degree has been awarded in accordance with UGC (Minimum standard and procedure for Awards of M.Phil/Ph.D. Degree) Regulation 2009.

Prof. Shakir Ali
Controller of Examinations

Copy to:

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7. Head, Department of Pharmacology
8. Supervisor, Dr. Mohd. Akhtar
9. Name of the Co-supervisor, Dr. Mohd. Mujeeb
10. Incharge, Web Cell for putting up on University's website
11. Editor, Spreadsheet, Jamia Hamdard
12. Mr. Md. Afroz Ahmad
13. Sr. P.A. to Vice Chancellor
14. File

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