Chemical Characterization, Analgesic, Antioxidant, and Anticholinesterase Potentials of Essential Oils From *Isodon rugosus* Wall. ex. Benth

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Isodon rugosus Wall. ex. Benth is an important species and is used in folk medicine for different types of pains such as abdominal pain, earache, toothache, gastric, and generalized body pain. Recently, we also have reported the antinociceptive potential of chloroform fraction of *I. rugosus*. In this research, we have investigated the antinociceptive, antioxidant and anti-cholinesterase potentials of essential oils from *I. rugosus* (Ir.EO), and have determined a possible mechanism of anti-nociception. The Ir.EO was subjected to gas chromatography-mass spectroscopy analysis to find out its chemical constituents. The Ir.EO was assayed for analgesic potential following acetic acid induced writhing, formalin test and hot plate method in animal models. The antioxidant activity was conducted against DPPH and ABTS free radicals following spectroscopic analysis. The cholinesterase inhibitory assays were performed using Ellman’s assay. The GC-MS analysis of Ir.EO revealed the identification of 141 compounds. Ir.EO demonstrated strong antinociceptive potential in all three in-vivo models. With the use of nalaxone, it was confirmed that the essential oil was acting on the central pathway of nociception. The Ir.EO also exhibited strong free radicals scavenging potential, exhibiting IC₅₀ values of 338 and 118 µg/ml for DPPH and ABTS free radicals respectively. In AChE and BChE inhibitory assays, the observed IC₅₀ values were 93.56 and 284.19 µg/ml respectively. The encouraging antinociceptive, antioxidant and anticholinesterase results revealed that Ir.EO is a rich source of bioactive compounds as obvious from the GC-MS results.

**Keywords:** essential oil, GC-MS, *Isodon rugosus*, antinociception, opioid receptors, antioxidant, anticholinesterase
INTRODUCTION

Globally, a large number of medicines are available for the treatment of pain and associated disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the management of pain and inflammation due to their strong efficacy (Zarin et al., 2005). However, their use is associated with severe side effects. Alternatively, the drugs from natural origins are considered to be relatively safe and are associated with fewer unwanted effects. Natural products, especially the plants play a vital role in the discovery of new chemical entities with potential therapeutic values (Rates, 2001; Ayaz et al., 2017a). The traditional use of plants is therefore a logical strategy to find out natural therapeutic agents for different ailments like pain and inflammation (Gupta et al., 2006). Despite the development of therapeutic agents for pain, there is still a demand to search out novel agents which could treat pain and related disorders more efficiently (Calixto et al., 2000).

The reactive oxygen species (ROS) are produced within the body as a result of redox processes and aerobic respiration. These ROS invade lipids, proteins, enzymes, DNA and RNA and ultimately damage the cells. These biomolecules play a vital role in stimulation, propagation and maintenance of inflammatory processes, as well as pain and neurodegenerative disorders (Zhu et al., 2004). These unwanted effects can be reduced by the use of antioxidants which either reduce the production of ROS or diminish them before reaction (Khalil et al., 1999; Cuzzocrea et al., 2001). In this regard, the essential oils isolated from herbal sources may be considered for the management of pain, inflammation, and free radicals scavenging. Several plants have been reported with strong antioxidant potentials against free radicals (Ahmad et al., 2015; Ayaz et al., 2015).

Alzheimer’s disease (AD) is a common neurodegenerative disorder characterized by cognitive hypo-function, behavioral turbulence and difficulties in life activities (Ali et al., 2017; Ayaz et al., 2017b). AD is believed to be the major cause of dementia in elder population (Ullah et al., 2016). According to the statistics, 27 million people are affecting globally from Alzheimer and is a major life threat after cancer and cardiovascular diseases (Hebert et al., 2003). AD pathogenesis include synaptic deficiency of essential neurotransmitter (acetylcholine, ACh) which is implicated in the neurotransmission (Sadiq et al., 2015). Other aspects of AD include accumulation of amyloid beta (Aβ), neurofibrillary tangles (NFTs), and free radicals induced neurodegeneration (McLean et al., 1999; Zeb et al., 2014a; Ahmad et al., 2016). The inhibition of cholinesterase is a vital biochemical target involved in the degradation of ACh which increases its accumulation in the synaptic region. Among the five clinically approved anti-Alzheimer drugs, four are cholinesterase inhibitors while the fifth drug memantine is glutamatergic system modifier. Despite the fact that several anti-amyloid and anti-NFTs drugs are in clinical trials, but, till date, no one is approved for clinical use. Furthermore, administration of free radical scavengers is also an important strategy, as Aβ is potent generator of free radicals and a mitochondrial poison. Plants are a source of mutli-potent drugs, including anti-AD drugs. Among the currently available anti-AD drugs, physostigmine, and galanthamine are derived from medicinal plants (Ahmad et al., 2016). Furthermore, natural products are free radicals’ scavengers and can be effective on multiple pathways (Ayaz et al., 2017c).

Isodon rugosus Wall. ex. Benth. is a well-known species of family Labiateae. The bark of I. rugosus is used ethnomedicinally in the treatment of dysentery and curing of body pain (Shuaib et al., 2015). Folklorically, the fresh leaves’ extract of I. rugosus is applied to the effected skin and is also used for earache (Sabeen and Ahmad, 2009). Moreover, the dried leaves of this plant can be used for the treatment of teeth pain (Akhtar et al., 2013). The plant has also been reported to posses potential effectiveness in gastric and abdominal pains (Ahmad et al., 2014). Moreover, other traditional uses of I. rugosus are attributed to its possible use against infectious diseases, pyrexia, blood pressure, rheumatism, and in pain associated with teeth (Khan and Khatoon, 2007; Adnan et al., 2012; Shuaib et al., 2014). The extracts of Isodon rugosus have been previously published to posses certain biological potentials like anti-diarrheal, analgesic, antimicrobial, anticholinesterase, antioxidant, cytotoxic, phytotoxic, hypoglycemic, and as bronchodilator (Sher et al., 2011; Ajmal et al., 2012; Janbaz et al., 2014; Zeb et al., 2014a,b, 2016, 2017).

Based on the ethnomedicinal importance and our previously published work, this piece of research is designed to investigate the in-vivo analgesic mechanism, in-vitro antioxidant, and anti-cholinesterase activities of essential oils of Isodon rugosus.

METHODS

Plant Sample Collection & Isolation of Essential Oil

Isodon rugosus was collected from Dir (L), KP, Pakistan in July. The name Isodon rugosus was confirmed by Dr. Ali Hazrat, Department of Botany, Shaheed Benazir Bhutto University Dir (U), KP, Pakistan. The plant sample was stored for future record at the herbarium with voucher specimen number 1016AZ. The essential oils were extracted by hydrodistillation with the help of a Clevenger type apparatus (Lambert et al., 2001). The isolated essential oils were stored in refrigerator.

Gas Chromatography Analysis

The phytocomponents of essential oils were separated using the same GC instrument as we previously reported (Ahmad et al., 2016). A capillary column having dimensions of 30 m × 0.25 mm with film thickness of 0.25 μm in combination with a flame ionization detector was used. The initial temperature was 70°C for 1 min, which was raised gradually to 180°C with 6°C/min increase for 5 min. Finally, the oven temperature was increased to 280°C with 5°C/min increase for 20 min. Temperature of the injector port was 220°C while that of detector was maintained at 290°C. Helium was used as a carrier gas. The sample was diluted in n-pentane (1/1,000, v/v) of 1 μl (Ayaz et al., 2016).

GC-MS Analysis

The GC-MS analysis of essential oil isolated from Isodon rugosus was determined with the previously reported parameters (Ayaz et al., 2015).
Identification of Components
The retention times and spectra of separated compounds by GC-MS were compared with the standard compounds for identifications. The mass spectrum of each separated compound with its fragmentation pattern was compared with the reported compounds (Stein et al., 2002; Adams, 2007).

Experimental Animals
The Swiss albino mice of either sex were used in analgesic experiments which were obtained from research laboratory of National Institute of Health, Islamabad, Pakistan. The animals were used as per the approval of the ethical committee, Department of Pharmacy, University of Malakand, Pakistan according to the animals Bye-Laws 2008 (Scientific Procedure Issue-1).

Acute Toxicity
Swiss albino mice were taken in various groups, having 5 test animals in each group. The essential oil samples were administered to the animals orally in different doses (250–2,000 mg/kg). To increase the aqueous solubility of essential oil, 0.1% v/v tween-80 (Sigma Aldrich)- was used. After administration of the doses, animals were critically observed for 72 h for hypersensitivity, abnormal behavior, and death. The experimental animals were observed for 20 days for sub-chronic effects and lethality (Hosseinzadeh et al., 2000).

Analgesic Activities
Acetic Acid-Induced Writhing Test
In acetic acid induced writhing test, the essential oil was administered orally (PO) in the same concentrations as mentioned in above section. After 30 min of interval, acetic acid (0.6%, 10 ml/kg) was injected into the mice intra-peritoneally. Tween-80 (0.5%, 3 ml/kg) was administered to Group I animals. The Group I was used as a negative control. The standard drug diclofenac sodium was administered to Group II with a dose of 10 mg/kg. The essential oil samples were administered to Groups III and IV in concentrations of 50 and 100 mg/kg respectively. After administration of acetic acid, the number of writhes were counted for 30 min (Franzotti et al., 2000).

Formalin Test
The formalin-induced licking test of Ir.EO was carried out using Swiss albino mice weighing 25–30 gm. The test was performed in a controlled environmental temperature (23 ± 2°C) with light-dark cycle of 12 h each. Food and water was freely available to the test animals throughout the investigations. The essential oil was administered intraperitoneally (I/P) to the experimental animals at various concentrations. After 30 min, 20 µl formalin (2.5%, v/v in distilled water) was injected subcutaneously (S/C) into the plantar surface of the hind paw. Tween-80 (0.5%, 3 ml/kg), a negative control in the experiment was administered to the Group I. Morphine (5 mg/kg), a standard drug, was administered to Group II animals. The animals in Groups III and IV were injected Ir.EO at concentrations of 50 and 100 mg/kg respectively. The nociceptive behavior was designated by formalin-induced licking of paw. The total time taken in the behavioral changes of the mice responses to nociception was recorded, such as licking and/or biting of the injected paw. The time taken was recorded for 30 min. The initial 5 min were considered as early phase, while 2nd period (15–30 min) as the late phase of the response. The early and late phase are termed as neurogenic and inflammatory phase, respectively (Sulaiman et al., 2008).

Hot Plate Test
The hot plate test method was assessed for the antinociception potential of essential oil isolated from I. rugosus as per the reported procedure (Zeb et al., 2016). In this method, a heated surface of a hot plate analgesia meter (Ugo Basile, model-7280) was maintained at 55 ± 0.2°C. The animals were kept over a heated surface in a closed glass cylinder. The time of the animals’ placement and licking of hind paw or jumping over the heated surface were recorded as response latency. These are the parameters as a result of the thermal reactions. The oil samples, in concentrations of 50 and 100 mg/kg, while morphine 5 mg/kg, i.p., were administered 30 min before the beginning of the assessment. Mice were observed before administration of samples, and then at 30, 60 and 90 min after the samples taken. The cut-off time was 20 s.

Involvement of Opioid Receptors
This experiment was carried out to confirm the possible involvement of opioid receptors in the essential oil-induced antinociception. The procedure was evaluated using a hot plate and formalin test method as mentioned earlier. In this method, different groups of experimental mice (n = 6) were pretreated with naloxone (5 mg/kg, S/C), which is a non-selective opioid receptor antagonist. Naloxone was injected 15 min before the administration of Ir.EO and morphine.

Antioxidant Assays
DPPH Assay
The DPPH free radicals scavenging effect was figured out for Ir.EO as previously published (Shah et al., 2015b). The DPPH solution (0.004%) in methanol was prepared which appeared with a deep violet color. Initially, the stock solution of essential oil with a known concentration of 1,000 µg/mL was prepared in ethanol. Then, this solution was diluted serially to obtain different concentrations from 62.5 to 1,000 µg/mL. Afterwards, 0.1 mL of the serially diluted concentration was added to 3.0 mL of DPPH solutions. This mixture was stored at dark place for 30 min at 23°C. After 30 min, the absorbance of each oil sample was measured by using double beam spectrophotometer at a wavelength of 517 nm. Ascorbic acid served as a positive control. The percent activity of all the samples was recorded as mean ± SEM. The percent radical scavenging potential was figured out using the following formula;

\[
\text{Scavenging effect} \% = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100
\]
ABTS Assay
Antioxidant potential of Ir.EO was also investigated using free radicals of 2, 2-azinobis [3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) (Ullah et al., 2017). Solutions of ABTS (7 mM) and potassium persulfate (2.45 mM) were prepared and mixed thoroughly. The prepared solution was stored in a dark place overnight to generate free radicals. The absorbance of this solution was adjusted at 745 nm to 0.7 by addition methanol (50%). ABTS solution 3 mL was added to the test tubes containing samples having volume of 300 µL. The solution was transferred to the sample holder and absorbance was recorded for 6 min by using a double beam spectrophotometer. Ascorbic acid was used as a standard. The percent ABTS free radicals scavenging potential of the oil sample was measured by using the given formula;

\[
\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of Ir.EO}}{\text{Absorbance of control}} \times 100
\]

Anticholinesterase Assays
Cholinesterases inhibitory potentials of Ir.EO was evaluated following Ellman’s assay (Ellman et al., 1961). This procedure is based on enzymatic breakdown of substrates like acetylthiocholine iodide and butyrylthiocholine iodide by AChE and BChE respectively to form 5-thio-2-nitrobenzoate anions. The resultant anions consequently form a complex with DTNB and are converted into UV detectable yellow color compound. The formation of this compound is quantified in the presence and absence of inhibitor agents. In brief, 5 µL enzyme solution was added to each well of micro plate with subsequent addition of 5 µL DTNB solution. The resulting mixture was incubated for fifteen min at 30°C in water bath, and finally 5 µL substrate solution was added to it. At the end, absorbances were recorded at 412 nm. The control samples were the same as above mentioned but were without inhibitors. The change in absorbance was observed beside reaction time. The activity of enzymes and its inhibitory activities were determined for control as well as test samples from the rate of absorption with change in time as, \( V = \Delta \text{Abs} / \Delta t \), and enzyme inhibition as;

\[
100 \times \frac{V}{V_{\text{max}}}
\]

Where, \( V_{\text{max}} \) is enzyme activity in the absence of inhibitor agent.

Estimation of IC\(_{50}\) Values
The median inhibitory concentration (IC\(_{50}\)) values of DPPH, ABTS, AChE, and BChE inhibitory assays were find out by linear regression analysis of the percent inhibition versus concentrations of the test samples through MS Excel program (Shah et al., 2015a; Sadiq et al., 2016).

Statistical Data Analysis
The values of all the tests were tabulated as mean ± S.E.M. Significant differences of the percent inhibitions of various test samples were analyzed via one way ANOVA following Bonferroni’s post-test using GraphPad Prism software in which the \( P < 0.05 \) were considered significant.

RESULTS

GC-MS Analysis
The essential oil of *Isodon rugosus* was subjected to GC-MS analysis and total of 141 compounds were identified. On the given GC method, the retention times of the identified compounds were from 6.057 to 81.661 min. The details of all identified compounds are given in Table 1.

Acute Toxicity
No mortality and behavioral change were observed at specified doses to confirm acute toxicity of the samples. According to the assay, dose up to 2,000 mg/kg was considered as safe for essential oil of *Isodon rugosus*.

Writhing Test
A dose dependent response was observed in acetic acid induced writhing test for the assessment of analgesic activity. The mean writhes of the standard drug at 10 mg/kg, was 21.83 ± 0.60 with 70.29% inhibition. The essential oil sample exhibited mean inhibition of 31.50 ± 1.28 with 57.14% at 100 mg/kg, while, at 50 mg/kg it exhibited mean inhibition of 41.00 ± 0.57 with 44.21%. At 100 mg/kg, Ir.EO and positive control exhibited a response of 57.14 and 70.29% respectively as shown in Table 2.

Formalin Test
The results obtained from formalin test are shown in the Table 3. The formalin injection (2%, i.p) to the animals revealed a typical biphasic licking response. In the control group, duration of licking was observed as 57.33 ± 0.88 and 67.00 ± 0.93 s for early (0–5 min) and late phase (15–30 min) respectively. Pre-treatment of mice with various concentrations of essential oil (50 and 100 mg/kg) produced a significant effect on the duration of licking in both phases. A dose of 100 mg/kg of Ir.EO brought a significant reduction in paw licking of 54.36 and 43.28% in early and late phase respectively. In comparison, Ir.EO in combination with naloxone exhibited 04.63 and 05.22% activity and 79.59% (late phase/inflammatory pain). The morphine in combination with naloxone demonstrated overwhelming reduction in both phases, i.e., 79.36% (early phase/neurogenic pain) and 79.59% (late phase/inflammatory pain). The morphine in combination with naloxone revealed 09.01% (early phase) and 07.95% (late phase) pain inhibitions. So, the naloxone reversed the antinociceptive effect of essential oil considerably at dose of 100 mg/kg in both phases as those of morphine.

Hot Plate Test
The results obtained in the hot plate assay are shown in Table 4. The Ir.EO revealed a dose dependent increase in the latency time as that of positive control. At 15 min, the mean reaction times for 50 and 100 mg/kg body weights of essential oil were observed as 06.40 ± 0.11 and 08.16 ± 0.08 min respectively. At 90 min, i.e., last interval, the mean reaction times of the same two doses were recorded as 04.40 ± 0.20 and 06.45 ± 0.07 min respectively. In comparison, the standard drug morphine exhibited reaction times of 12.41 ± 0.11 and 09.38 ± 0.08 min at initial and last interval respectively.
**TABLE 1** | List of all the compounds identified in the GC-MS analysis of essential oil of *Isodon rugosus*.

| S. No. | Compound label | RT   | Name            | Formula | Hits (DB) |
|-------|----------------|------|-----------------|---------|-----------|
| 1     | Methyl ethyl ketone | 6.057 | Butanone       | C4H8O   | 10        |
| 2     | 2-cyclohexenyl vinyl ether | 6.683 | Na              | C8H12O  | 10        |
| 3     | Alpha-Copaene    | 18.689 | Alpha Copaene  | C15H24  | 10        |
| 4     | BETA-bOURBONENE  | 19.522 | BETA. BOURBONENE | C15H24  | 10        |
| 5     | 8-Isopropyl-1-methyl-5-methylene-1,6-cyclodecadiene | 19.605 | Germacrene D | C15H24  | 10        |
| 6     | 2,6-Dimethylocta-1,4,7-triene | 19.643 | Cis-Achillene   | C10H16  | 10        |
| 7     | 2-Butanone, 4-(2,2-dimethyl-6-methylenecyclohexyl) | 19.764 | Na              | C13H22O | 10        |
| 8     | Bicyclo[3.3.1]non-2-ene, 7-oxa-2,6,9-trimethyl-5-acetoxymethylene | 19.866 | Na              | C14H22O3 | 10        |
| 9     | 1,4-Dimethylpent-2-nylbenzene | 19.976 | Na              | C13H18  | 10        |
| 10    | Beta-Caryophyllen | 20.312 | Caryophyllene  | C15H24  | 10        |
| 11    | 1,1,7-TRIMETHYLCYCLOHEXANE-1H-CYCLOPROPANE| 20.584 | AROMADENDRENE | C15H24  | 10        |
| 12    | Alpha-Cubebeene  | 20.851 | Alpha Cubebeene | C15H24  | 10        |
| 13    | CADINA-1,4-DIENE | 20.937 | Na              | C12H14N2O | 10        |
| 14    | 3,8-Dimethylundecane | 20.98  | Na              | C13H28  | 10        |
| 15    | Cyclosolongifolene, 8,9-dehydro- | 21.184 | Na              | C13H22  | 10        |
| 16    | Epi-bicyclosesquiphellandrene | 21.227 | Na              | C15H24  | 10        |
| 17    | Alpha-Amorphene   | 21.477 | Alpha Amorphene | C15H24  | 10        |
| 18    | Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl | 21.549 | Ar-Curcumene    | C15H22  | 10        |
| 19    | 7-Methoxy-1,2,3,4-tetrahydro-9H-pyrido[3,4-d]pyridine | 21.788 | Na              | C12H14N2O | 10        |
| 20    | Cadina-4,9-diene  | 22.197 | Na              | C12H26O | 10        |
| 21    | 2,3-Dimethylfuran | 22.452 | Germacrene D-4-ol | C15H26O | 10        |
| 22    | 4-Methyl-6,7-dihydrobenzene | 22.603 | Calamene        | C15H22  | 10        |
| 23    | 2,3-Dimethylfuran | 22.755 | Na              | C13H22  | 10        |
| 24    | 5-(1-Methylethyl)-1,2-naphthalenedione | 22.823 | Na              | C10H14O | 10        |
| 25    | 5H-Inden-5,5-diene, 1,2,3,3a,4,7a-hexahydro-7a-methyl- | 22.917 | CALACORENE     | C13H16  | 10        |
| 26    | 3-Heptadecan-5-yn | 23.126 | Na              | C17H30  | 10        |
| 27    | cis-(4,4a,5,5a-Trans-1,2,3,4-tetrahydro-5H-cyclopenta[c]pyridine | 23.471 | Trans-Nerolidol | C15H26O | 10        |
| 28    | 6-Heptadecan-9-yn | 23.885 | Na              | C15H26  | 10        |
| 29    | Verbenene        | 23.923 | Verbenene       | C10H14  | 10        |
| 30    | N-Butyl-3-hydroxybutyramide | 24.061 | Na              | C8H17NO | 3         |
| 31    | N-(1-Methylethyl)-2-(1-methylethyl)benzamide | 24.073 | Na              | C13H19NO | 10        |
| 32    | Clov-2-ene-9-alpha-ol | 24.145 | Na              | C15H24O | 10        |
| 33    | VIRIDIFLOROL     | 24.221 | Veridiflorol    | C15H26O | 10        |
| 34    | Ledol            | 24.319 | Ledol           | C15H26O | 10        |
| 35    | 1H-Inden-1-one, octahydro-, cis | 24.462 | Hydrindan      | C9H14O  | 10        |
| 36    | 3-Hydroxy-4-methyl-5,6,7,8-tetrahydroquinoline | 24.531 | Na              | C10H13NO2 | 3         |
| 37    | 1,2-Naphthalenedione, 3,8-dimethyl-5-(1-methyl) | 24.561 | Mansoneone C    | C15H16O2 | 10        |
| 38    | Humulene-1,6-dien-3-ol | 24.618 | Na              | C15H26O | 10        |
| 39    | Cedr-8-ene      | 24.696 | Alpha-cedrene  | C15H24  | 10        |
| 40    | Longifolenealdehyde | 24.781 | Longifolaldehyde | C15H24O | 10        |
| 41    | 2,3-Bis(adamantylcarbonyl)ethynylbicyclo[2.2.1]hepta-2,5-diene | 24.993 | Na              | C33H36O2 | 10        |
| 42    | 1,2-Diacetyl-4-methylbenzene | 25.134 | Na              | C11H12O2 | 10        |
| 43    | 1,2-Diacetyl-4-methylbenzene | 25.392 | T-Muurolol     | C15H26O | 10        |
| 44    | 4,4-Dimethylpentanenitrile | 25.406 | Na              | C7H13N  | 1         |
| 45    | Cadin-4-en-10-ol | 25.57  | Alpha-Cadinol  | C15H26O | 10        |
| 46    | 5,7-Dimethoxyquinoline | 25.583 | Na              | C11H11N | 10        |
| 47    | Azulene, 1,4-dimethyl-7-(1-methyl) | 25.686 | Azulon         | C15H18  | 10        |
| 48    | 1,4-Methanobenzocyclohexene, 1,2,3,4,4a,5,8,9,12,12a-decahydro- | 25.884 | Na | C15H22 | 5         |
| 49    | 4a,5,6,7,8-Hexahydro-4a-methyl-2(3H)-naphthalenone | 26.157 | Na              | C11H16O | 10        |
| S. No. | Compound label | RT  | Name                     | Formula        | Hits (DB) |
|-------|----------------|-----|--------------------------|----------------|-----------|
| 52    | 7,7-dichlorobicyclo[3.2.0]hept-2-en-6-one | 26.262 | Na                      | C15H24O        | 10        |
| 53    | 6-Methylenebicyclo[2.2.1]hept-2-en-1-ol | 26.348 | Na                      | C8H11O         | 1         |
| 54    | MUUROLA-4,10(14)-DIEN-3-ONE | 26.374 | Na                      | C15H22O        | 10        |
| 55    | Pentalene, octahydro-1-(2-octyldecyll) | 26.513 | Na                      | C26H50O        | 10        |
| 56    | 1.beta.,4.beta.H,10.beta.H-Guaia-5,11-diene | 26.627 | Gamma.-Gurjunene       | C15H24O4       | 10        |
| 57    | Phosphorochloridic acid, diethyl ester | 26.788 | Diethyl phosphorochloridic acid, diethyl ester | C4H10O3        | 10        |
| 58    | Isosipulegyl acetate | 26.902 | Na                      | C12H20O        | 10        |
| 59    | 1,3-dimethyl-3-acetoxyethyl-2-oxabicyclo[2.2.2]octan-5-one | 27.314 | Na                      | C12H18O4       | 10        |
| 60    | Benzyl benzoate | 27.873 | Na                      | C14H12O4       | 10        |
| 61    | Trans-1-(3''-Cyclopropyldiene)propan-1'-yl]-1-[(propen-3''-yl)cyclopropane | 28.23 | Na                      | C12H20O        | 10        |
| 62    | Ethyl 3-phenylhexa-2,4-dienoate | 29.066 | Na                      | C14H16O2       | 10        |
| 63    | 1-(8'-Methoxy-7'-methyl-1',2',3',4'-tetrahydronaphthalen-1'-yl)ethanol | 29.312 | Na                      | C14H20O4       | 10        |
| 64    | 6-(p-Tolyl)-2-methyl-2-heptenol | 29.584 | Nuciferol               | C15H24O9       | 10        |
| 65    | 2-Butanol, 4-[2,2,6-trimethylcyclohexyl]-, acetate | 30.058 | Tetrahydroxyacetone     | C15H28O4       | 10        |
| 66    | 1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl | 30.327 | Patchoulane             | C15H28O4       | 10        |
| 67    | Trans-Pinocarvyl acetate | 30.688 | Na                      | C12H20O        | 10        |
| 68    | 1H-[R,S],[R,S],[R,S]-2,2-dimethyl-1-[(R,S,S,S)-hydroxypropyl]-6-(R,S)-n-octyl-... | 31.48 | Na                      | C18H32O4       | 10        |
| 69    | 3-Angelate of felicol | 31.704 | Na                      | C20H34O3       | 10        |
| 70    | 2-thia-6-methyl-7-[2-formylmethyl]bicyclo[3.2.0]hept-6-ene-2,2-dioxide | 31.795 | Na                      | C10H14O2       | 10        |
| 71    | Aphanamol | 32.079 | Aphanamol               | C14H20O4       | 10        |
| 72    | 5,9-Undecadien-2-one, 6,10-dimethyl | 32.317 | Geranylacetone          | C13H22O        | 10        |
| 73    | 4-Chlorobutyric acid, octadecyl ester | 32.639 | Na                      | C22H43O2       | 10        |
| 74    | Bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl- | 32.976 | Na                      | C15H24O9       | 10        |
| 75    | 2,9-Dimethyl-8-oxatetracyclo[5.4.1.1(3,10).0(5,9)]tridecane-2,6-dioxide | 33.499 | Na                      | C14H20O4       | 10        |
| 76    | 1-(4-Hydroxy-3-isopropenyl)-4,7,7-trimethyl-cyclohept-1-enyl-ethanol | 33.918 | Na                      | C15H24O9       | 10        |
| 77    | 1,2-pentanediol, 5-[(6-bromodehydroxy)-2-hydroxy-2,5,5a,8a-tetramethyl-1-napht… | 34.228 | Na                      | C20H35O3       | 10        |
| 78    | 1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cyclocadene | 35.087 | Na                      | C15H26O        | 10        |
| 79    | Viridiflorene | 35.376 | Viridiflorene           | C15H24O        | 10        |
| 80    | Dodecylicholine | 35.978 | Dodecylicholine        | C26H56O2       | 10        |
| 81    | Sec-Butyl 2,3,4,6-tetra-methyl-D-galactopyranoside isomer | 36.292 | Na                      | C14H28O4       | 10        |
| 82    | 1,3-EPIMANOLYL OXIDE | 36.366 | Epimanol oxide         | C20H34O        | 10        |
| 83    | Pentadecane | 36.435 | Pentadecane            | C15H32O        | 10        |
| 84    | Cyclocadene, octyl | 36.599 | Octylcyclocadene       | C18H28O        | 10        |
| 85    | Labd-14-ene, 8,13-epoxy-, (13R)- | 37.211 | Na                      | C20H34O        | 10        |
| 86    | 5.alpha.-ally-6.alpha.-hydroxy-5.beta.-beta.-dimethyl-trans-decalin-1-one | 37.726 | Na                      | C15H24O2       | 10        |
| 87    | Naphthalene, 7-buty1-1-hexyl | 38.181 | Na                      | C20H28O        | 10        |
| 88    | 1,1,7,12-tetramethyl-8-ethyl-1,2,3,4,9,10,11,12-octahydrophenanthrene | 38.718 | Na                      | C20H30O        | 10        |
| 89    | Benzamidine, 4-(4-pentylphenyl)- | 39.442 | Na                      | C18H22N2       | 10        |
| 90    | 3-Eicosene | 39.652 | Na                      | C20H40O        | 10        |
| 91    | Eicosane | 39.652 | Na                      | C20H40O        | 10        |
| 92    | Sulfurous acid, hexyl nonyl ester | 40.208 | Na                      | C15H32O3       | 10        |
| 93    | 2-Cyclohexen-1-ol, 3-methyl-6-[1-methylethyl] | 40.372 | Na                      | C10H18O        | 10        |
| 94    | 1-Acetyl-2-amino-3-cyano-7-isopropyl-4-methylazulene | 40.546 | Na                      | C17H18N2O       | 10        |
| 95    | 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl | 40.682 | Phytol                  | C20H40O        | 10        |
| 96    | 1,1,8,9A-tetramethyl-2,3,5,6,7,9a-hexahydro-1h-benzo[a]cycloheptene | 40.915 | Na                      | C15H24O        | 10        |
| 97    | 2-n-Heptylcyclopentanone | 41.419 | Na                      | C12H22O        | 10        |
| 98    | 7,11,15-TRIMETHYL-3-METHYLENE-1-HEXADEcene | 41.645 | Neophytadiene          | C20H36O        | 10        |
| 99    | Stearic acid | 42.294 | Stearic acid            | C18H36O2       | 10        |
| 100   | Abietyl alcohol, dehydro | 42.844 | Abietyl alcohol, dehydro | C20H36O        | 10        |
Moreover, the recorded mean reaction time for Ir.EO with naloxone (50:1 mg/kg) at 15 min was 05.28 ± 0.13 min. Similarly, for morphine and naloxone (5:1 mg/kg), the mean reaction time observed was 04.35 ± 0.13 min at initial 15 min. In our experiment, we found a distinct reduction in reaction time with the administration of naloxone.

### Involvement of Opioid Receptors

In both hot plate and formalin models, we noticed that Ir.EO revealed a similar activity as that of morphine. The potency of Ir.EO was reduced effectively by opioid antagonist naloxone. With the use of naloxone, the decreased in reaction time in hot plate method and reversing the paw licking in formalin assay confirmed the possible involvement of opioid receptors.
Antioxidant Assays

The antioxidant potential of Ir.EO using DPPH and ABTS free radicals scavenging methods are shown in Table 5.

DPPH Assay

The observed percent inhibitions for Ir.EO using DPPH free radicals was 63.67 ± 1.20, 56.67 ± 0.67, 46.33 ± 0.33, 41.33 ± 0.88, and 33.00 ± 1.15% at concentrations of 1,000, 500, 250, 125, and 62.5 µg/ml respectively. The calculated IC_{50} value from the dose response curve was 338 ± µg/ml. In comparison, the standard drug ascorbic acid exhibited 89.00 ± 1.73, 86.33 ± 1.45, 83.00 ± 1.73, 79.67 ± 0.88, and 77.33 ± 0.88% inhibitions at 1,000, 500, 250, 125, and 62.5 µg/ml respectively with an IC_{50} value of <0.1 µg/ml.

ABTS Assay

In ABTS assay, Ir.EO attained 64.33 ± 0.88, 62.00 ± 0.57, 56.67 ± 1.20, 51.00 ± 0.57, and 39.67 ± 1.76% inhibitions at 1,000, 500, 250, 125, and 62.5 µg/ml respectively. The calculated IC_{50} for Ir.EO in scavenging ABTS free radicals was 118 µg/ml. In this assay, ascorbic acid demonstrated 91.33 ± 0.88, 85.67 ± 0.33, 81.67 ± 0.67, 78.00 ± 1.15, and 75.00 ± 0.57% inhibitions at 1,000, 500, 250, 125, and 62.5 µg/ml respectively attaining an IC_{50} value of <0.1 µg/ml.

Cholinesterase Inhibition Assay

In AChE inhibitory assay, Ir.EO exhibited concentration dependent inhibitions against the enzymes (Table 6). Ir.EO showed 67.50 ± 1.04% AChE inhibition at 1.0 mg/ml concentration with IC_{50} of 93.56 µg/ml. Similarly, the observed inhibitory potential against BChE at the same tested concentration as AChE was 61.33 ± 0.67% with an IC_{50} of 284.19 µg/ml. In comparison, the standard drug galanthamine exhibited 0.371 and 3.324 µg/ml IC_{50} against AChE and BChE respectively.

DISCUSSION

In our designed work, the essential oil of *I. rugosus* was evaluated for antinociceptive, antioxidant, and anticholinesterase potentials. The essential oils of plants are sources of wide variety of bioactive compounds (Dehpour et al., 2009). The pharmacological potentials of essential oil can be attributed to the hydrophilic

**TABLE 2** | Percent anti-nociceptive potential of essential oil following acetic acid induced writhing model.

| Samples | Dose (mg/kg) | Mean writhes | % Analgesic activity |
|---------|--------------|--------------|----------------------|
| Negative cont. | – | 73.50 ± 0.61 | 0.00 |
| Ir.Eo | 50 | 41.00 ± 0.57*** | 44.21 |
| Ir.Eo | 100 | 31.50 ± 1.28*** | 57.14 |
| Positive cont. | 10 | 21.83 ± 0.60*** | 70.29 |

Ir.Eo, Essential oil isolated from *Isodon rugosus*; Mean writhes are represented as mean ± SEM. ***P < 0.001.

**TABLE 3** | Effect of Essential oil of *Isodon rugosus* on formalin induced pain in mice.

| Samples | Dose (mg/kg) | 0–5 min | % Inhibition | 15–30 min | % Inhibition |
|---------|--------------|---------|-------------|-----------|-------------|
| Negative cont. | – | 57.39 ± 0.88 | – | 67.00 ± 0.93 | – |
| Ir.Eo | 50 | 39.50 ± 1.11*** | 31.10 | 49.67 ± 1.92*** | 25.86 |
| Ir.Eo | 100 | 26.16 ± 0.94*** | 54.36 | 38.33 ± 0.71*** | 43.28 |
| Mor | 5 | 11.83 ± 1.24*** | 79.36 | 13.67 ± 0.67*** | 79.59 |
| Mor + Nal | 5 + 1 | 54.67 ± 1.02<sup>ns</sup> | 04.63 | 63.50 ± 1.17<sup>ns</sup> | 05.22 |
| Ir.Eo + Nal | 50 + 1 | 52.16 ± 0.70<sup>ns</sup> | 09.01 | 61.67 ± 1.14<sup>ns</sup> | 07.95 |

Ir.Eo, Essential oil of *Isodon rugosus*; Mor, Morphine; Nal, Naloxone; Total time is represented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

**TABLE 4** | Effect of Essential oil of *Isodon rugosus* on hot plate induced pain in mice.

| Samples | Dose (mg/kg) | Reaction time on hot plate |
|---------|--------------|---------------------------|
| | 15 min | 30 min | 45 min | 60 min | 90 min |
| Negative cont. | – | 04.51 ± 0.10 | 02.43 ± 0.14 | 03.55 ± 0.07 | 02.11 ± 0.15 | 03.20 ± 0.15 |
| Ir.Eo | 50 | 06.40 ± 0.11 | 04.18 ± 0.11 | 05.76 ± 0.08 | 04.81 ± 0.14 | 04.40 ± 0.20 |
| Ir.Eo | 100 | 08.16 ± 0.08 | 06.81 ± 0.13 | 07.91 ± 0.11 | 06.80 ± 0.07 | 06.45 ± 0.07 |
| Mor | 5 | 12.41 ± 0.11 | 11.63 ± 0.06 | 11.36 ± 0.08 | 10.45 ± 0.07 | 09.38 ± 0.08 |
| Mor + Nal | 5 + 1 | 04.35 ± 0.13 | 02.80 ± 0.19 | 04.35 ± 0.07 | 02.78 ± 0.10 | 03.56 ± 0.14 |
| Ir.Eo + Nal | 50 + 1 | 05.28 ± 0.13 | 03.31 ± 0.08 | 04.70 ± 0.08 | 03.11 ± 0.15 | 03.70 ± 0.06 |

Ir.Eo, Essential oil of *Isodon rugosus*; Mor, Morphine; Nal, Naloxone; Total time is represented as mean ± SEM.

**TABLE 5** | Effect of Essential oil of *Isodon rugosus* and standard drug galanthamine on formalin induced pain in mice.

| Samples | Dose (mg/kg) | Reaction time on formalin induced pain model (min) |
|---------|--------------|-----------------------------------------------|
| Negative cont. | – | 04.51 ± 0.10 |
| Ir.Eo | 50 | 06.40 ± 0.11 |
| Ir.Eo | 100 | 08.16 ± 0.08 |
| Mor | 5 | 12.41 ± 0.11 |
| Mor + Nal | 5 + 1 | 04.35 ± 0.13 |
| Ir.Eo + Nal | 50 + 1 | 05.28 ± 0.13 |

Ir.Eo, Essential oil of *Isodon rugosus*; Mor, Morphine; Nal, Naloxone; Total time is represented as mean ± SEM.
nature of its components and the same nature of our body cell membranes (Ait-Ouazzou et al., 2011). Various components of essential oils can easily get distributed to different compartments of our body including the central nervous system (Lambert et al., 2001; Vyas et al., 2008). In our current investigational study, the antinociceptive potential of essential oil was recorded with significant results. The possible mechanism of antinociceptive activity of Ir.EO was figured out as the central pathway due to involvement of opioid receptors. Recently, we have also reported the antinociceptive potential of chloroform fraction of *I. rugosus* following the same mechanism. The antinociceptive potential of essential oil may be due to the presence of large number of bioactive compounds as obvious from its GC-MS analysis. Among the identified compounds, we also observed some of the bioactive compounds previously reported with analgesic potentials. These compounds include α-copaene, germacrene D, β-caryophyllene, α-caryophyllene, aromadendrene, calamene, viridiflorol, mansonone C, t-muurolol, α-cadinol, azunol, phytol, neophytadiene, and simvastatin. In short, α-copaene has been reported to possess strong analgesic and antioxidant potentials (Kim et al., 2008; Chen et al., 2011; Costa et al., 2011). Likewise, germacrene D also possesses analgesic and antioxidant effects (Del-Vecho-Vieira et al., 2009; Victoria et al., 2012). β-Caryophyllene is also reported with its analgesic and antioxidant potentials (Calleja et al., 2013; Klaue et al., 2014). The antinociceptive activity of aromadendrene has also been demonstrated (Cruz et al., 2011). Similarly, α-caryophyllene has been reported for the treatment of body inflammatory pain (Pianowski et al., 2004). The analgesic and antioxidant effects of calamene have also been demonstrated with significant results (Azevedo et al., 2013; Imam et al., 2014). Viridiflorol, a well-known bioactive compound is also reported to possess analgesic and radical scavenging potentials (Perry et al., 1997; do Amaral et al., 2007). Moreover, Mansonone C (including its reduced form) is also responsible for direct antioxidant activity (Villamil et al., 1990). T-muurolol has been verified for inhibitory activity against DPPH free radicals (Cheng et al., 2004). The analgesic activity of α-cadinol has also been reported with notable results (Boutaghan et al., 2011). The antinociceptive aspects of azunol has also been published previously (Ushiyama et al., 2009). In the same way, ledene has also been reported to possess analgesic activity (Alagammal et al., 2012). A well-known compound, i.e., phytol, is famous for its antioxidant potential along with its antinociceptive potential (Santos et al., 2013). Neophytadiene is also among the famous analgesic and antioxidant candidates (Jayashree et al., 2015). Similarly, simvastatin is also previously reported with its analgesic and antioxidant potentials (Carneado et al., 2002; Chen et al., 2013).

Literature review and the results of our current investigations go parallel with sound correlation. The traditional use of *I. rugosus* as analgesic is efficiently verified in the current research project, along with the identification of bioactive compounds. Beside the antioxidant potential of Ir.EO, we also evaluated its AChE and BChE inhibitory potentials. Among other pathological targets of Alzheimer disease, inhibitions of cholinesterase and free radicals are also vital targets. Among the clinically approved anti-Alzheimer drugs, four are cholinesterase inhibitors, which signify the importance of this target in the symptomatic management of the disease. In the current study, we observed a moderate in-vitro cholinesterase inhibitory activity of Ir.EO. In AChE and BChE inhibitory assays, Ir.EO showed concentration dependent inhibitions against the enzymes with IC50 values of 93.56 and 284.19 µg/ml respectively. Though the in-vitro enzyme inhibitory activity of essential oil was low in comparison to galanthamine, yet, we hypothesize that it will have more availability at the target site. However, further studies are required regarding in-vivo efficacy of our tested essential oil.

**CONCLUSION**

Based on the literature survey regarding the medicinal aspects of *I. rugosus* and the results of current investigational study, it

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**TABLE 5 | Antioxidant activity of essential oil of *Isodon rugosus* at various concentrations.**

| Test sample | Free radicals | Conc. 62.5 µg/ml | Conc. 125 µg/ml | Conc. 250 µg/ml | Conc. 500 µg/ml | Conc. 1,000 µg/ml | IC50 µg/ml |
|-------------|---------------|-----------------|----------------|----------------|----------------|------------------|-------------|
| EO          | DPPH          | 33.00 ± 1.15*** | 41.33 ± 0.88*** | 46.33 ± 0.33*** | 56.67 ± 0.67*** | 63.67 ± 1.20*** | 338         |
| EO          | ABTS          | 39.67 ± 1.76*** | 51.00 ± 0.57*** | 56.67 ± 1.20*** | 62.00 ± 0.57*** | 64.33 ± 0.88*** | 118         |
| A.A         | DPPH          | 77.33 ± 0.88    | 79.67 ± 0.88    | 83.00 ± 1.73    | 86.33 ± 1.45    | 89.00 ± 1.73    | <62.5       |
| A.A         | ABTS          | 75.00 ± 0.57    | 78.00 ± 1.15    | 81.67 ± 0.67    | 85.67 ± 0.33    | 91.33 ± 0.88    | <62.5       |

EO, Essential oil; A.A, Ascorbic acid. **P < 0.001.

**TABLE 6 | Anticholinesterase activity of essential oil of *Isodon rugosus* at various concentrations.**

| Test sample | Enzymes | Conc. 62.5 µg/ml | Conc. 125 µg/ml | Conc. 250 µg/ml | Conc. 500 µg/ml | Conc. 1,000 µg/ml | IC50 µg/ml |
|-------------|---------|-----------------|----------------|----------------|----------------|------------------|-------------|
| E.Oil       | AChE    | 42.30 ± 0.47    | 56.46 ± 1.27    | 57.00 ± 0.57    | 62.67 ± 0.88    | 67.50 ± 1.04     | 93.56       |
| E.Oil       | BChE    | 36.58 ± 0.97    | 41.95 ± 2.01    | 49.87 ± 1.67    | 56.00 ± 1.15    | 61.33 ± 0.67     | 284.19      |
| Gal         | AChE    | 74.00 ± 1.00    | 79.66 ± 1.85    | 85.00 ± 1.73    | 87.46 ± 1.79    | 94.83 ± 1.92     | <62.5       |
| Gal         | BChE    | 63.83 ± 0.92    | 71.16 ± 0.92    | 77.83 ± 1.09    | 83.16 ± 1.42    | 88.00 ± 1.25     | <62.5       |

Data is expressed as Mean±SEM; Gal and E.Oil are abbreviated for Galanthamine and Essential oil respectively.
may be deduced that the essential oil of *I. rugosus* is a good source of natural bioactive compounds containing numerous analgesic and antioxidant agents. Its antioxidant potentials along with cholinesterase inhibitory activity will be potentially effective in the management of Alzheimer's disease patients. It may also be inferred that further exploitation of essential oil of *I. rugosus* may lead to the development of new analgesic and/or anti-Alzheimer drug candidates.

**AUTHOR CONTRIBUTIONS**

AZ and SA carried out experimental work, data collection and literature search under the supervision of AS. FU helped as co-supervision of the research work. MA, NM and UR drafted the manuscript for publication. AS supervise the overall project and make the final version of publication. All the authors have read and approved the final manuscript for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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