WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.453
Volume 13, Issue 11, 1860-1874. Review Article ISSN 2277–7105

IN-VITRO ASSAYS TO INVESTIGATE ANTI-INFLAMMATORY, ANTI-DIABETIC AND ANTI- MICROBIAL ACTIVITY OF HERBAL EXTRACT OF ARISTOLOCHIA INDICA- A REVIEW

Neethu R.*, Tanishka Tandel, Niyatee Thakur, Aamir Ahmad, Ruchi Borkar and Dr. Rupesh Pingle

Assistant Professor, Department of Pharmacology, NCRD's Sterling Institute of Pharmacy, Nerul, Navi Mumbai, Maharashtra, India.

Article Received on
18 April 2024,

Revised on 08 May 2024,
Accepted on 28 May 2024

DOI: 10.20959/wjpr202411-32576

***Corresponding Author**
Mrs. Neethu R.
Assistant Professor, Department of Pharmacology, NCRD's Sterling Institute of Pharmacy, Nerul, Navi Mumbai, Maharashtra, India.

ABSTRACT

Aristolochia, a diverse genus within the Aristolochiaceae family, boasts over 400 perennial species. Traditionally, various Aristolochia plants have been used in herbal remedies for snakebites, wounds, and tumors, with some applications persisting, especially in Chinese medicine. Locally known as Isharmul, this endangered medicinal plant, characterized by its unique long, twining stems, grows in the plains and low hills spanning Nepal's lower Bengal to Bangladesh's Chittagong and India's Coromandal Coast. Aristolochia indica Linn, part of the Aristolochiaceae family, was traditionally utilized in indigenous medicine to treat skin ailments, inflammation, and as a purgative. It possesses antimicrobial, antioxidant, antibacterial, antiinflammatory, immunomodulatory, anticancer, antidiabetic, and antivenom properties Studies have been interested in the Aristolochia indica had revealed that the methanolic extract from the plant contains number of phenols and flavonoids. So, the Aristolochia indica is rich source of nature which can be accounted for traditional uses as antioxidant the aristolochic acid is the main chemical complex of the plant. This acid contributes carcinogenic and genotoxic property and other effects of the plant. Aristolochia indica is a rich natural source due to its traditional use as aristolochic acid. The chemical component of the plant acts as a classic defense mechanism against various infections, as the antioxidant aristolochic acid is the exclusive chemical constituent of the plant. Aristolochia

indica has traditionally been used to treat various conditions, including respiratory diseases, gastrointestinal diseases, and skin infections.

KEYWORDS: Aristolochic Acid, Protein Denaturation Assay, Alpha Amylase, Agar Well.

INTRODUCTION

From the delicate Asarum to the enigmatic Hydnora, the Aristolochiaceae family encompasses seven fascinating genera: Asarum, Saruma, Lactoris, Hydnora, Prosopanche, Thottea, and Aristolochia. Focusing on tropical and temperate areas, America, Asia, and Australia host roughly 550 known species. Locally known as Isharmul, this endangered medicinal plant, characterized by its unique long, twining stems, grows in the plains and low hills spanning Nepal's lower Bengal to Bangladesh's Chittagong and India's Coromandal Coast. Aristolochia indica Linn, part of the Aristolochiaceae family, was traditionally utilized in indigenous medicine to treat skin ailments, inflammation, and as a purgative.

Its flowers are greenish white, arranged in auxiliary cymes, while its rounded oblong fruits contain numerous winged compressed seeds within six chambers. Additionally, it is utilized as an abortifacient, antiseptic, antineoplastic, emmenagogue, antibacterial, anti-inflammatory and inhibitor of phospholipase A. Due to its significant medicinal value, Aristolochia is employed in Ayurveda and indigenous medicine. It possesses antimicrobial, antioxidant, anti-venom, immunomodulatory, anti-diabetic, anti-cancer, and anti-inflammatory properties. Natural germination is challenging due to low seed potency, leading to its listing as a rare and endangered species in regions like Assam, Madhya Pradesh, and the Eastern Ghats of India.

Fig: 1.1 Aristolochia Indica Plant

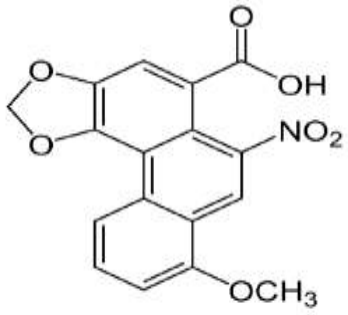


Fig 1.2 Aristolochic acid

MORPHOLOGY

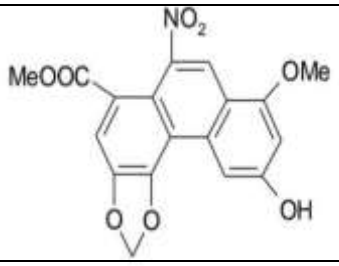
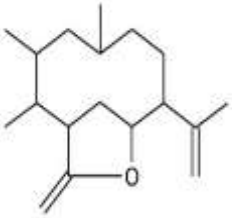
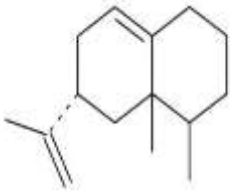
A. indica is characterized by long and twisted stems and is a perennial shrub or herbaceous plant. The flowering and fruiting periods of this vine generally fall from December to February. The leaves are arranged alternately, with a smooth edge, with wavy curves on the sides, heart-shaped at the base with pointed or obovate ends.[18] These vines reach a height of 10 to 50 cm. The flowers are in the leaf axis and have a spherical base that merges into a long perianth. This tube ends in a brightly coloured tongue-shaped petal.[31] The fruit ripens in an elongated capsule decorated with six longitudinal veins. Each capsule contains elongated seeds with side wings. Fruit formation lasts all year round. The diagnostic characters are trained leaves at the base and elongated morphology, which distinguish this species from its relatives.[32]

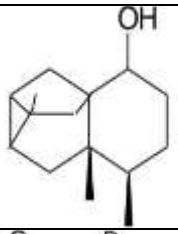
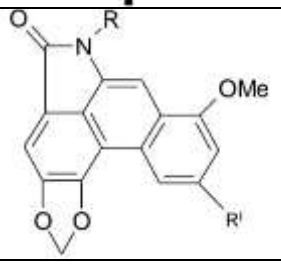
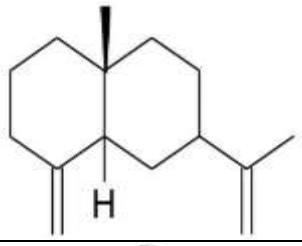
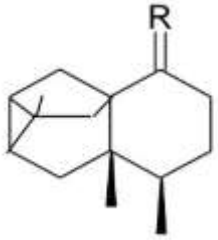
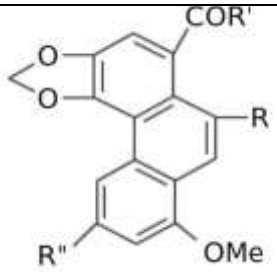
Aristolochic acid

Aristolochic acid (AA), a mixture of aristolochic acid I (AAI) and aristolochic acid II (AAII), is found in members of the Aristolochiaceae family and is commonly used in traditional medicinal plants.[33] People exposed to aristolochic acid, which is found in some medicinal plants used in traditional medicine, have a significantly increased risk of developing severe nephropathy as well as urological, hepatobiliary, and possibly other cancers.[34] Products containing AA are banned or subject to restrictions in numerous countries. However, in the USA and Europe, AA herbal supplements are readily available online.[35,36,37] The carcinogenic properties of AA are due to its irreversible properties, which require the implementation of appropriate measures to limit exposure and reduce the risk of cancer. The World Health Organization (WHO) raised concerns about aristolochic acid in 2001. Following this, in 2002, the International Agency for Research on Cancer (IARC) classified plants containing aristolochic acid as Group 1 carcinogens for the first time.[37]

Phytochemical work reported on aristolochia indica

The roots yielded two novel sesquiterpene hydrocarbons named ishwarane and aristolochene, while the structure of a tetracyclic sesquiterpene called ishwarone was identified.[38,39] Researchers discovered ishwarol in the roots and determined its structure.[38] They also isolated a new sesquiterpene hydrocarbon, named 5β-H, 7β, 10α-selina-4(14), II-diene, and characterized it.[40] The research yielded a bounty of interesting molecules, including five novel phenanthrene derivatives (II-VI). Additionally, stigmast-4-en-3-one and sitosterol were isolated, along with two unidentified sesquiterpene alcohols with remarkably high melting points of 1030°C and 1500°C.[41] A novel sesquiterpene with the structure (12S)-7, 12-Secoishwaran-12-o' was identified and characterized in a recent study.[42] Isolation of Aristololactam N-β-D-glucoside, a phenanthrene derivative, and it two steroids, 3β-hydroxy-stigmast5-en-7-one and 6β-hydroxy-stigmast-4-en-3-one, was reported from A. indica.[43] The roots harbor a variety of compounds including aristolindiquinone, aristolide, 2-hydroxy-1-methoxy-4H-dibenzoquinolone-4,5-(6H)-dione, cephradione, aristolactam IIa, stigmastenones II and III, methylaristolate, β-sitosterol-β-D-glucoside, aristolactam glycoside I, ishwarol, ishwarone, methylaristolate, and aristolochene.[44,45] A new naphthoquinone named Aristolindiquinone, along with Aristolochic acids and Aristolactams, was discovered in A.indica.[46,47]

Chemical name	Chemical structure
Aristolochic acid	
Aristolactone	
Aristolochene	

Ishwarol	
Aristolochic acid D me ether lact am (R=H, R^1 = OMe) **Aristolactam-β-D glucoside** (R=Glu, R^1 = H)	
5β-H, 7 β, 10*α*-Selina-4(14),11-diene (A new sesquiterpene hydrocarbon)	
Ishwarane (R= H) **Iswarane** (R= O)	
New Phenanthrene Derivatives a) R,R^{II} = H, R^1 = OH b) R,R^{II} = H, R^1 = NH_2 c) R=H, R^{II}= OMe d) R=NO_2, R^1 = Me, R_{II} = H	

Anti inflammatory action

Studies interested in the Aristolochia indica had stated that the methanolic extract from the plant contains number of phenols and flavonoids contents.[1,2] Final compounds are chemical constituents present in plants which are classic compounds to protect the plants from animals and different types of infections.[9] Though traditionally used as an antioxidant due to its abundance of natural compounds, Aristolochia indica contains aristolochic acid, a well-known carcinogen.[8] This acid contributes to carcinogenic and genotoxic property of the plant and other effects of the plant. Plant possesses the antibacterial, immunomodulatory, and anti-inflammatory activity too along with anti-inflammatory activity.[1,8]

Inflammatory diseases are prevalent worldwide. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly employed to prevent and manage these conditions, but they can lead to adverse effects such as hemorrhage and ulcers, which pose risks to human health. Therefore, in vitro assessments of plants are conducted globally to evaluate their anti-inflammatory properties, aiming to mitigate and prevent the adverse effects associated with NSAIDs.[2]

Assay methods

1. Protein denaturation test
2. Membrane stabilization method
3. Assay of proteinase inhibition

1. Protein denaturation test

The main purpose of ovalbumin denaturation test is intended to determine whether the active ingredients or compounds can stop or hinder the formation of ovalbumin degradation by exposing it too the aristolochic acid.[4] Denaturation is a term used to describe protein undergoes structuring changes and loses its specific biological activity in the presence of heat, pH or other denaturing agents.[4] The conformation of protein is disrupted and changed by denaturation with the help of denaturation agents and change in physical properties lead to loss of functionality. The ovalbumin denaturation test is used to measure a drug or the ability of the compound to prevent or reduce ovalbumin denaturation to evaluate its anti-inflammatory effects. The ovalbumin denaturation test is based on the principle that stabilize protein structures are stabilize by the drug and prevent denaturation.[3] Denaturation of ovalbumin assay could potentially be possible to detect anti-inflammatory properties. Inflammation is thought to be a denaturation of proteins. Compound prevents the denaturation of proteins and inhibits the COX enzyme. Test sample can be incubated with ovalbumin solution.[5] Then % inhibition is calculated by, %inhibition = (Absorbance of control- Absorbance of sample) X 100/ Absorbance of control.[3]

2. Method of membrane stabilization

Inflammation can cause lysis of the lysosomal membrane, releasing enzymatic components that cause breaking down the biological polymers like proteins which can lead to various disorders. Mechanism of action of non-steroidal anti-inflammatory drug is to inhibit lysosomal enzymes or to stabilize lysomal membrane.[3] Exposure to harmful substances can damage red blood cells in several ways. This damage can lead to lysis (rupture) of the cell

membrane, a process known as hemolysis. Additionally, the hemoglobin within the red blood cell may become oxidized.[6] Harmful substances include a hypotonic environment like physical agent heat, and chemical agents like methyl salicylate, and phenyl hydrazine. The anti-inflammatory potential of a substance can be evaluated based on its ability to protect red blood cell membranes. Since these membranes share similarities with lysosomal membranes, which are involved in inflammation, inhibiting both the breakdown (lysis) of red blood cells caused by heat and the decrease in blood pressure (hypotension) can be indicative of anti-inflammatory activity.[5] A hypotonic solution causes excess fluid to absorb into the red blood cells and increase osmotic pressure which leads to rupture the cell membrane. A damaged red blood cell membrane makes the cell susceptible to secondary damage from lipid peroxidation by free radicals. Leakage of lysosomal components from cell leads to further inflammation and tissue damage after extracellular release of lysosomal enzymes. The stability of cell membranes caused by extracts can be assessed using two methods: heat-induced hemolysis and hypotonic solution-induced hemolysis. These tests can be conducted with human, rat, or mouse red blood cells.[3] The erythrocyte membrane serves as an analogue to the lysosomal membrane. Therefore, the effect of the extracts on stabilizing erythrocytes is utilized to stabilize the lysosomal membrane.[7]

Calculate the %inhibition: %inhibition = (Absorbance of control- Absorbance of sample) X 100/ Absorbance of control [3]

3. Proteinase inhibition assay

Standard drug which is use as anti-inflammatory drug is Ketotifen. Studies have shown that the petroleum ether and ethanol root extracts of A. indica significantly inhibit mast cell degranulation, almost as potently as the standard drug ketotifen. Ethanol root extract of plant Aristolochia indica at a dose of 150 mg/kg reduced rat paw swelling by 70%, showing strong anti-inflammatory activity. Antidote Aristolochia indica has anti-inflammatory, analgesic, and antipyretic properties.[3] A. indica, the dried plant extract was tested for the venom of Heteropneustes fossilis. A reduction in inflammation was observed after co-administration of the plant extract and ichthyocrinotoxin venom to male albino rats treated with carrageenan (Das et al. Aristolactam I and (−)hinokinin exerted potent anti-inflammatory effects and inhibited the production of cytokines TNF-α and IL-6 in LPS-stimulated THP-1 cells.[10,12]

Percentage inhibition = (Abs control –Abs sample) X100/ Abs control.[11]

Anti microbial action

Aristolochia indica (Linn) belong to family Aristolochiaceae. The Aristolochia indica is prosperous source of nature which can be accounted for traditional uses as aristolochic acid.

Aristolochia indica contains ethanolic extract which contains several numbers of alkaloids, flavonoids, glycosides, steroids, and tannins. Chemical constituent of the plant acts as classic defence mechanism against various infections as antioxidant aristolochic acid is the primary chemical constituent of the plant. The plant has a property of genotoxicity, carcinogenetic properties. Aristolochia indica has been traditionally used for the treatment of numerous ailments, including respiratory illness, gastrointestinal disturbances, and skin infections. Antimicrobial action suppresses the growth of bacteria and their ability to reproduce.[22,24]

Antimicrobial activities of aristolochia indica

Several studies have been reported about the antimicrobial activities of Aristolochia indica against an extensive range of microbial pathogens, including bacteria, fungi, and viruses. Antimicrobial efficacy of Aristolochia indica extracts and isolated compounds has been demonstrated through various in vitro and in vivo studies. Extracts from various parts of the plant, such as stems, roots, seeds, and leaves, have shown promising antimicrobial properties. The antimicrobial activity of Aristolochia indica is attributed to its rich phytochemical composition, including alkaloids, flavonoids, phenolic compounds, and terpenoids. These bioactive constituents exert their antimicrobial effects through multiple mechanisms, such as disruption of microbial cell membranes, inhibition of microbial enzymes, and modulation of microbial gene expression.[23,24]

Antimicrobial activity of Aristolochia indica was determined by

1. **Agar well method:** The agar well diffusion method is a common technique used to assess the antimicrobial activity of plant extracts or microbial cultures. The method involves first evenly spreading a microbial inoculum (a standardized culture of microorganisms) across the surface of an agar plate. Then, a sterile cork borer or tip is used to aseptically create a well (a small hole) 6 to 8 millimeters in diameter within the agar. A specific volume (typically 20-100 microliters) of the antimicrobial agent or extract solution at the desired concentration is then loaded into the well. Finally, the agar plate is incubated under conditions suitable for the growth of the test microorganism. If the agent or extract possesses antimicrobial activity, it will diffuse through the agar and inhibit the growth of

the surrounding microorganisms. This inhibition zone, a clear area devoid of microbial growth, is then measured to determine the potency of the antimicrobial agent.[25]

2. **Minimum inhibitory concentration:** The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that effectively stops a microorganism from growing visibly after a defined incubation period. It is typically expressed in milligrams per liter (mg/L) or micrograms per milliliter (µg/mL). MIC is determined using a broth dilution method. In this method, Mueller-Hinton broth and Sabouraud Dextrose Broth are commonly used for bacterial and fungal strains, respectively. The inoculated culture plates are incubated at specific temperatures: 37°C ± 2°C for 24 hours for bacteria and 27°C ± 2°C for 48 hours for fungi. Antimicrobial activity is then evaluated by measuring the diameter of the clear zone (inhibition zone) around the well containing the antimicrobial agent.[24]

3. **Disk diffusion method:** The widely utilized disk diffusion method was used to evaluate the antibacterial qualities of natural antimicrobial agents and botanical extracts. Developed in 1940, this testing technique is the official method employed in many clinical microbiology labs for routine assessment of antimicrobial susceptibility. In this process, agar plates are inoculated with a standardized amount of the test microorganisms. Then, filter paper discs, each 6mm in diameter and carrying the desired concentration of the test substance, are positioned on the agar surface of petri dishes. These dishes are subsequently incubated under appropriate conditions.[25]

Anti-diabetic action

Diabetes mellitus is a metabolic disorder characterized by hyperglycemias, glycosuria, hyperlipidemia, Negative nitrogen balance and sometime ketonaemia. Two significant types of diabetes mellitus are:

Type 1: Insulin dependent diabetic mellitus (IDDM)/ juvenile onset diabetes mellitus.

Type 2: Non-Insulin dependent diabetic mellitus (NIDDM)/ maturity onset diabetes mellitus.[1]

Diabetes mellitus is the most widespread chronic disease globally, affecting a staggering 25% of the population. Currently, it impacts 150 million people, with projections estimating this number to reach 300 million by 2025. In diabetes, reactive oxygen species (ROS) are generated which causes lipid peroxidation and membrane damage and these free radical lead

to produce secondary complication likes kidney damage, nerve and eye damage. Hence herbal drug is increasing popular for the treatment of the diabetic mellitus due to efficacy and less side effect. Aristolochia indica also known as india birthwort (family- Aristolochiaceae) which is a medicinal plant contain beneficiary effect especially Anti- Venom, Anti- bacterial, Anti- inflammatory and Anti- diabetic effect.[27]

Assays of Invitro Anti-diabetic Activity

1. **Alpha amylase enzyme assay (DNS Method):** The alpha amylase assay was performed using miller's method, i.e. the DNS method. This method is a redox reaction where DNS is reduced by reducing sugar to 3-amino-5-nitro salicylic acid in alkaline medium.[3] Acarbose standard drug was used as an inhibitor. It is a reversible competitive inhibitor. Acarbose binds to the active site of alpha amylase enzyme hence it inhibits half of enzyme activity. When the enzyme activity decreases, the formation of product also decreases therefore the intensity of the color is reduced.[3] Alpha amylase inhibitory assay of Aristolochia indica methanolic extract were increasing with the increasing concentration. It was concluded that the methanolic extract exhibits higher alpha amylase inhibitory activity at 60.12% inhibition at the concentration 300µg/ml. Which is compare with the standard drug acarbose which exhibits 82.60% inhibition.[4] The percentage inhibition was calculated by the following formula:

% inhibition =[(O.D of control – O.D of test sample)/O.D of control]×100.[27,28]

2. **Alpha glucosidase enzyme assay:** Alpha glucosidase is a carbohydrate hydrolase enzyme which is widely distributed on the brush edge of the small intestine mucosa. It can hydrolyze the sugar Compounds into monosacharides, oligosaccharides, that leads to increase in postprandial glucose level. Inhibition of alpha glucosidase activity slow down the carbohydrate digestion activity led to reduce the glucose absorption into the blood and controlling blood sugar level.[5] The inhibitor used was the standard drug acarbose. The alpha-glucosidase inhibitory activity of the methanolic extract of Aristolochia indica increased with higher concentrations. It was observed that the methanolic extract demonstrated greater alpha-glucosidase inhibitory activity, reaching 57.28% inhibition at a concentration of 400µg/ml. This was compared to the standard drug acarbose, which exhibited 77.18% inhibition. The percentage inhibition was calculated by the following formula:

% inhibition = [(O.D of the control – O.D of test sample)/ O.D of control] × 100.[27,30]

CONCLUSION

The Aristolochia genus, a diverse family with over 400 perennial species, is the focus of this article. We primarily highlight various in vitro studies conducted on Aristolochia indica, including investigations into its anti-inflammatory, anti-diabetic, and anti-microbial properties. We propose minimizing animal usage in in vitro assays due to ethical concerns and the importance of human welfare. Many reviewers have utilized NSAIDs like aspirin and diclofenac as standards for assessing anti-inflammatory activity, and acarbose as a standard for evaluating anti-diabetic activity.

ACKNOWLEDGEMENT

The Authors convey their sincere gratitude to Department of Pharmacology, NCRD's Sterling Institute of Pharmacy, Nerul, Navi-Mumbai for their co-operative support to carry out the review work in this Institute.

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47. Fig 1.1 https://commons.wikimedia.org/wiki/File:Aristolochia_indica_05.jpg
48. Fig 1.2 https://en.wikipedia.org/wiki/Aristolochic_acid