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"PHYTOCHEMICAL SCREENING OF SEED EXTRACT OF CROTALARIA JUNCEA WITH RESPECT TOANTIFERTILITY AGENTS."

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Abstract
The present work with HPLC investigated the properties of ethinylestradiol, diosgenin and saponins. *Crotolaria juncea* contains a mixture of various types of saponins. Further studies are recommended in various parts of the plants so as to isolate and characterize the bioactive compounds. The present investigation helps the discovery of plant-based drugs to human welfare. Further purification, identification and characterization of the bioactive chemical constituent's compounds would be our priority in future studies.

Introduction

Plants have served as a natural source of antifertility substances. Henshaw (1953) listed many plants used by primitive people in different countries to control fertility. Though many indigenous plants have been shown to prevent births, only a few plants have so far been investigated for anti-spermatogenic activity (Kholkute, 1977; Segal, 1985; Rao, 1988; Murugavel and Akbarshah, 1991; Chatterjee et al, 1994; Raji and Bolarinwa, 1997; Madhusudan Reddy et al, 1997; Naseem et al, 1998; Purohit, 1999). Crotalaria juncea Linn. (Papilionaceae), commonly known as Sunn hemp, is used Indian Ayurvedic Medicine states where in its various parts have properties like analgesic, astringent, emmenagogue, abortifacient and also treatment of skin diseases. It is also mentioned that the seeds are known for various medicinal properties (Kirtikar and Basu, 1935; Wealth of India, 1952; Chopra et al, 1956). The seeds of this plant have been reported to possess antifertility activity (Chaudhury, 1966; Bala and Garg, 1973). The alcoholic extract of C. juncea seeds has shown antiimplantation activity in mice (Prakash et al., 1993). In the present work an attempt has been made to find plant metabolite with respect to antifertility agents, which in future could be used as a female antifertility agent.

Objectives

1. To analyze plant metabolite with respect to antifertility agents.
2. To compare the metabolites from plants and contraceptive pills.

Material and Method

The plant material was collected, identified and the extract was prepared for phytochemical analysis. The methods are given in the following sections.

Collection of Plant Material

On the basis of literature and survey of trible people (Gaykar *et al*., 2006) following plants were selected for the scientific study of spermicidal activity. The selected plants were *Crotalaria juncea* L., (Fabaceae).Seed samples were collected from various areas of Ahmednagar District, India in their natural habitat. They were identified from Botanical Survey of India, Western Circle, Pune (Annexure I). The voucher specimens were deposited in the Herbarium, BSI, Pune as well as Herbarium of Department of Botany, New Arts, Commerce and Science College, Ahmednagar. (ABK 004).

Morphology of *Crotalaria juncea*

Crotalaria juncea, known as **brown hemp**, **Indian hemp**, **Madras hemp**, or **sunn hemp**, is a tropical Asian plant of the legume family (Fabaceae). It is generally considered to have originated in India.It is now widely grown throughout the tropics and subtropics as a source of green manure, fodder and lignified fiber obtained from its stem. Sunn hemp is also being looked at as apossible bio-fuel. It can be an invasive weed and has been listed as a noxious weed in some jurisdictions. It bears yellow flowers and elongate, alternate leaves.

Extraction of the Plant Materials

The seed were air dried at room temperature followed by pulverization to powder form using mortar and pestle. The powdered seeds were subjected to aqueous extraction as well as extraction of active components from seed powder was performed with petroleum ether by using Soxhelet. Polar and non-polar solvent were taken into consideration for extraction. Solvent of each sample was removed by vacuum rotary evaporator at room temperature. The remaining residues were collected and preserved at 4 for further experiment. The non-polar Petroleum ether was used which being more effective than methanol extracts (Magahi, 2015), so the extracts were made in this non polar solvent.

Habit of *Crotalaria juncea* L.

***Crotalaria juncea* L. Seeds**

Preliminary qualitative phytochemical screening

The preliminary phytochemical analysis of the aqueous and petroleum ether extracts were performed for testing different chemical groups present in seed extracts of selected traditional contraceptive plants using standard procedures by Sofowora A. (2008), Harbone (1973) and Trease Evans (2010).

Test for alkaloidsMayers test

About 1ml of each extract was stirred with 2ml of 1% aqueous HCl on a steam bath andfiltered. 1ml of the filtrate was treated with a few drops of Mayers reagent Formation of white or pale yellow precipitate indicates the presence of alkaloids.

Wagner's test

2ml of aqueous and petroleum ether extract was acidified with 1.5% v/v of hydrochloricacid and a few drops of Wagner's reagent was added. Formation of yellow or brown precipitate indicates the presence of alkaloids.

Hager's test

2mg of the aqueous and petroleum ether extract taken in a test tube, a few drops of Hager's reagent was added. Formation of yellow precipitate confirms the presence of alkaloids.

Dragendorff's test

2 ml of the petroleum ether extract, 5ml of distilled water was added and 2M Hydrochloric acid was added until an acid reaction occurs. To this solution 1ml of Dragendorff's reagent was added. Formation of orange or orange red precipitate indicated the presence of alkaloids.

Test for flavonoidsShinodas test

0.5 ml of the aqueous and petroleum ether extract, magnesium turnings were added followed by diluted HCl. Formation of pink, reddish or reddish-brown colour indicated the presence of flavonoids.

Alkaline reagent test

2 ml of aqueous and petroleum ether extract, few drops of sodium hydroxide solution was added. At first intense Yellow color was appeared which turned colorless on addition of diluted HCl indicated the presence of Flavonoids.

Test for glycosidesBorntragers test

The 5 ml of each aqueous and petroleum ether extracts were boiled separately with equal amount of dilute sulphuric acid in a test tube for 5 minutes. It was filtered and cooled. The filtrate was measured and equal amount of chloroform was added, shaken for gently. It was kept for 2 minutes. The lower layer of chloroform was separated. The layer was stunned with half of its volume by dilute ammonia. Appearance of rose pink tored color indicated the presence of glycoside.

Test for triterpenoids Liebermann-Burchards test

Few drops of acetic anhydride were added to 2ml of aqueous and petroleum ether extract. The mixture was heated up to boiling, cooled and then 1ml of concentrated sulphuric acid was added in both extracts along the sides of the test tubes. Formation of a violet colored ring indicated the presence of Triterpenoid.

Salkowski test 1ml of extract was dissolved in chloroform and few drops of concentrated sulphuric acid were added to it. Formation of reddish brown colour on the inner face suggested the presence of Terpenoids.

Test for Saponins- Frothing test

2ml of extract was vigorously shaken with distilled water and was allowed to stand for10 min. Formation of constant froth indicate the presence of saponins

. **Test for Steroids Liebermann-Burchard's test**

Few drops of acetic anhydride were added to 2 ml of aqueous and petroleum ether extract. The

mixture was heated up to boiling, cooled and then 1 ml of concentrated sulphuric acid was added in both extracts along the sides of the test tubes. Formation of green colored ring indicated the presence of steroids.

Salkowski test

1ml of extract was dissolved in chloroform and few drops of concentrated sulphuric acid were added to it. Red colour indicated the presence of steroids.

Gas Chromatography-Mass Spectrometry Analysis:

GC-MS analysis:

It was carried out using a Hewlett Packard gas chromatography (model 6890 series) equipped with a flame ionization detector and Hewlett Packard 7633 series indicator, MS transfer line temperature of 250?. The GC was equipped with a fused silica capillary column HP-5MS (30 × 0.25mm), film thickness 1.0µm. The oven temperature was held at 50 for 5 mins holding time and raised from 50 to 250 at a rate of 2/min, employing helium gas (99.99%) as a carrier gas at a constant flow rate of 22cm/sec. 1.0 micron of extract (1mg dissolved in 1ml absolute alcohol), at a split ratio of 1 : 30 was injected. MS analysis was carried out on Agilent. Technology network Mass spectrometer (model 5973 series) coupled to a Hewlett Packard Gas Chromatography Model 6890 series) equipped with NIST08 Library software database. Mass spectra were taken at 70 ev/200 scanning rate of 1 scan/sec.

Identification of compounds

Interpretation of mass spectrum of the unknown component was conducted by comparing the mass spectra with the spectrum of the known components stored in the data system National Institute Standard and Technique library (NIST-2008, Turbo mass Ver. 5.4.2). The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The name, molecular weight, structure and mass fragmentation of the components of the test materials were given.

HPLC analysis method

Preparation of standard solution

10mg of standard diosgenin was weighed and dissolved in 5ml of methanol by means of sonicated for 15 min. The solution was diluted up to 10ml with methanol (1mg/ml). 1ml solution was taken out from stock solution and diluted up to 10 ml with methanol (100µg/ml). The ethinylestradiol and saponin were extracted effectively by alkaline hydrolysis followed by HPLC-UV determination.

HPLC instrumentation

The details of the instrument used were Shimadzu Prominence HPLC modular system, Binary Gradient with PDA detector with Shim-pack solar ^{18}C column (Chromolith Chro- matography, A Japan Company). It was a reverse phase (stationary phase was non-polar and mobile phase was polar).

The column temperature was 40 maintained for all samples. The mobile phases were composed of acetonitrile and water in the proportion of 90:10. The adjustable experimental variables were the conditions of gradient modes and mobile phase compositions. The flow rate was kept at 1ml/minute.

The injection volume was 25µl. Ethinyl estradiol was detected using absorbance at PDA multilevel 225nm, 4nm, while diosgenin and saponins were detected using absorbance at PDA multilevel 203nm, 4nm. Optimization of HPLC conditions as a standard procedure was carried out prior to analysis.

Origin and Role of Phytochemicals of Crotalaria Juncea L.

The medicinal importance of plants is due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, Saponins, Steroids, and Tannins etc. The preliminary phy- to chemical tests performed were of qualitative and quantitative. The selected plants for the study were used by the ethnic and tribal people as traditional contraceptives in Ahmednagar District (Kadam *et al*., 2013). So these plants were analyzed to find out the novel phytochemicals which may be responsible and correlate with contraception.The results of the phytochemical analysis revealed varying constituents of these extracts. Further some phytochemicals were screened by GC- MS and HPLC technique.

GC-MS analysis

The gas chromatograms of the seed extract which showed 46 distinct peaks of various compounds were identified by GC-MS. Major five peaks of compounds were iden- tified through the NIST Library database.

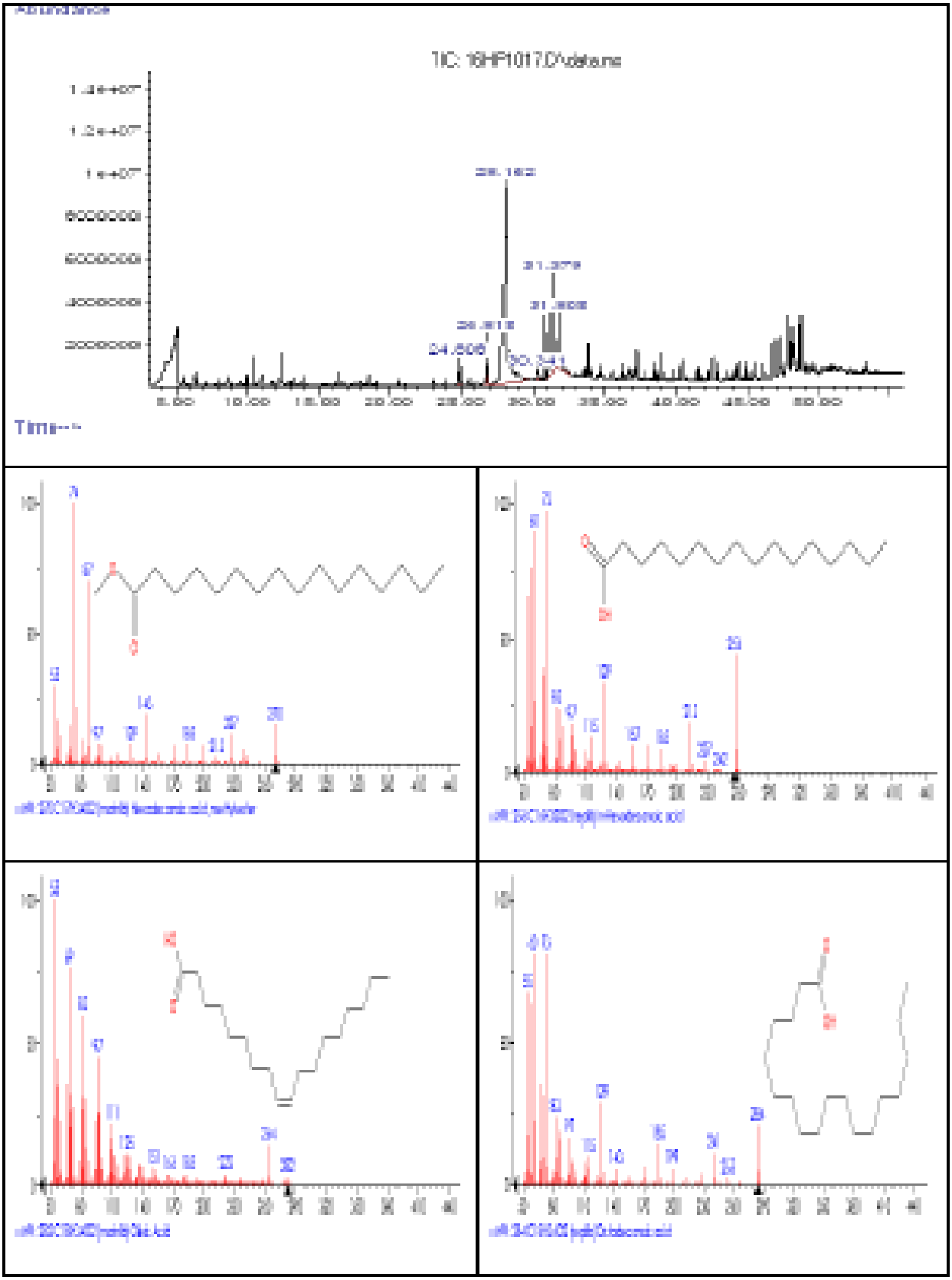
The major compounds present in the Petroleum ether seed extract of Crotolaria were hexadecanoic acid methylester amounted (2.789%), n-hexadecanoic acid (68.314%), oleic acid (1.051%), octadecanoic acid (6.876%). The major contribution is of hexadecanoic acid which has medicinal value.

The structural and kinetics studies showed that the fatty acid, n-hexadecanoic acid, is an inhibitor of phospholipase, hence, an anti-inflammatory compound (Aparna *et al*., 2012).

The work on GC-MS analysis of *Crotolaria juncea* L. seeds is scanty. Some of the observations were made by few workers. It has been reported that sunn hemp seeds contain various pyrrolizidine alkaloids such as junceine, riddelliine, senecio- nine, seneciphylline and trichodesmine (Smith and Culvenor, 1981).

The amounts of pyrrolizidine alkaloids reported by Ji *et al*., (2005) were small that agrees with other reports. This was confirmed again by Nurhayati and Ober (2005) who did not detect alkaloids in cotyledons, leaves, flowers or roots of *C. juncea* L., which suggests that alkaloid production is limited to the seeds.

Crotolaria juncea L. belongs to a genus that is known for the production of toxic dehydropyrrolizidine alkaloids (seeds were estimated to contain 0.15% w/w.), extracts of the roots, stems, leaves and seeds (Colegate, 2012). The present study showed the availability of different kind of chemical compounds which were not reported by any worker. These results are newly reported in this work.



GC-MS Chromatographs of Chemical composition of *Crotalaria juncea* L. seed extract.

Result and Conclusion

The percentage yields of seed extracts of C. juncea were 9.3% and 7.4% (w/w) with brown and yellowish color, respectively. The seed of C. juncea were used to investigate primarily phytochemical studies. The selected parts of plant were analyzed for phytochemical screening forthe extracts obtained from Soxhelet extraction successfully using petroleum ether. The extracts were subjected to various qualitative tests for phyto-constituents such as alkaloids, flavonoids, triterpenoids, tannins, steroids and glycosides.

The extracts of seed of C. juncea reacted positively with Dragendroff's reagent test, which showed a white precipitate indicating the presence of alkaloids. The extracts have shown positive response to Salkowski test.The formation of yellow colored lower layer indicated presence of triterpenoids, and the extracts have shown positive response to alkaline reagent test. The disappearing of the yellow color indicated the presence of flavonoids and the extracts have also shown positive response to the dilute ferric chloride.

Plants have been a source of medicine in the past centuries and today scientists and the general public recognize their value as a source of new or complimentary medicinal products. Recently, wide array of research investigations highlight the potential health beneficial principles from phytal sources. Medicinal plants constitute one of the main sources of new pharmaceuticals and health care products. There has been an increase in demand for the phytopharmaceuticals all over the world because of the fact that the allopathic drugs have more side effects.

Various phytoconstituents like alkaloids, flavonoids, tannins, xanthones, triterpenes, quinonesetc. were involved in anti- fertility activity (Kadam,2017).

Although a number of plants have been reported to possess cent percent antifertility activity but till date these plants have not yet come up at the level of clinical trials. Standardization of methods, quality control, data on safety and efficacy need for proper understanding of the use of herbal medicines.

The present study provides evidence that solvent extract of plants contains medicinally important bioactive compounds and this justifies the use of plant species as traditional medicine for treatment of various diseases.

Present study concludes that various phytochemical were present in seeds of selected traditional contraceptive plants. These phytochemicals were alkaloids, flavonoids, glyco-sides, triterpenoids, saponins and steroids. The saponin and steroidal compounds were the major compounds in *Crotolaria juncea* L. was devoid of Triterpenoids.

GC-MS analysis of the Petroleum ether plant extract led to the identification of different compounds. Hexadecanoic acid found in Crotolaria seeds was the new report, may be antifertility activity

The present work with HPLC investigated the properties of ethinylestradiol, diosgenin and saponins. *Crotolaria juncea* contains a mixture of various types of saponins.

Further studies are recommended in various parts of the plants so as to isolate and characterize the bioactive compounds. The present investigation helps the discovery of plant-based drugs to human welfare. Further purification, identification and characterization of the bioactive chemical constituent's compounds would be our priority in future studies.

Significance of Research Work

The sources of many of the new drugs and active ingredients of medicines are derived from natural products. Bioassay-guided fractionation method is commonly employed in drug discovery research due to its effectiveness to directly link the analyzed extract and targeted compounds using fractionation procedure that followed with certain biological activity

The present investigation will be helps for discovering new source as contraceptive and also informed the methods and modes of contraceptives with optimal effectiveness but with minimum side effects. The rural and tribal women are well known with wild resources around them which

having abortifacient or contraceptives properties, but it is very difficult to identifying quality and effectiveness of such plants.

The study will also helpful to create alternative cheaper, herbal source against artificial contraceptive without side effects. The selected active compounds will be useful for formulation of drugs in near future which will be free from various side effects.

In our recent study we observed that, artificial contraceptives do exhibit side effects in certain cases. Now days, plant products are more popular than the synthetic drugs. Natural contraceptives offer alternatives for women who have problem with modern contraceptives options, particularly women living in rural areas in India. Hence there is need for searching suitable product from indigenous medicinal plants that could be effectively used in place of synthetic hormonal contraceptives.

In view of these points, present investigation will be avail natural contraceptives which having without long term side effects, cheaper and safer as compared to artificial contraceptives. For investigation of correct and reliable source for contraception, it is necessary to study biologically active botanical substances or fertility-regulating agents of plant origin which eco-friendly in approach and interfere with the natural patterns of reproduction.

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