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Phytochemical constituents of different extracts of *Azadirachta indica* leaves in urine solvent of non-pregnant cow

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Abstract

This study includes qualitative analysis of phytochemicals present in fresh urine of non-pregnant cow, photoactivated urine of non-pregnant cow, urine distillate of non-pregnant cow, photoactivated cow urine extract of *Azadirachta indica* and cow urine distillate extract of *Azadirachta indica*. The different extracts of *Azadirachta indica* leaves were prepared by using non-pregnant cow urine as solvent. The results of qualitative phytochemical analysis showed that different extracts of *A. indica* leaves contain phytochemicals viz. Alkaloids, Glycosides, Tannins, Proteins, Reducing sugars, Phytosterols, Phenolic compounds, Saponins.

Thus, presence of these phytochemical suggests the pharmacological potential of the wonder tree "neem". It was concluded that non-pregnant cow urine extract of *A. indica* leaves contain pharmacologically active constituents.

Keywords: *Azadirachta indica*, Ayurveda, cow urine, herbal extracts, phytochemicals

1. Introduction

In developing countries like India, the herbal medicine is still the backbone of about 75-80% of the world population for primary health care. This is primarily because of the general belief that herbal drugs are without side effects being cheap and locally available. The use of herbal remedies throughout the world exceeds that of the conventional drugs by two to three times [1]. The World Health Organization (WHO) defines traditional medicine is a health practice, approaches, knowledge and belief including plant, animal and mineral based medicines, manual technique, spiritual therapies and exercises, applied singularly or in combination to treat, diagnose and prevention of illness and maintain well-being of human and animal health (WHO). Ayurveda is the oldest and most valuable source of unique natural products [2]. Since ancient times medicinal plants have been used to combat various diseases. As they are non-toxic, cheap, readily available and effective [3]. Therefore, after going through the various literatures on medicinal plants has revealed that there are many plants which have different pharmacological properties. The *Azadirachta indica* is one amongst them.

Azadirachta indica is a fast-growing evergreen popular plant belonging to the family Meliaceae commonly known as Neem. It grows well in tropical countries and is used as a therapeutic agent for treating various ailments in the Indian culture as a part of traditional medicine [4]. Numbers of studies have confirmed that *A. indica* leaves extracts possesses insecticidal, antiviral, antifungal, and antibacterial properties [5]. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex [6]. Phytochemicals are the natural bioactive compounds found in plants. These phytochemicals are primarily classified into two classes, i.e primary and secondary constituents, according to their functions in plant metabolism [7]. Plant chemicals are regarded as secondary metabolites because the plants that manufacture them have little need for them. They are synthesized in all parts of the plant body; bark, leaves, stem, root, flower, fruits, seeds etc. i.e. any part of the plant body may contain bioactive components [8].

In spite of the fact that the cow urine has great pharmacological importance and has great aesthetic and medicinal value through its utility has been mentioned in holy texts of Indian literature [9]. Cows were regarded as wealth and were the backbone of the economy of ancient Indians. Cattle were one of the most frequently used animals described in Vedas. Cows are regarded as mother ("Gau-mata") and referred to as Aghnya. Cattle husbandry was well developed during the Rigvedic period (1500-1000 BC) and the cow (Kamdhenu) was adored and considered the 'best wealth' of mankind [10].

Cow urine has a unique identity in Ayurveda and has been described in “Sushruta Samhita” and “Ashtanga Sangraha” to be the most effective substance secretion of animal origin with innumerable therapeutic values. Cow urine has been recognized as water of life or “Amrita” (beverages of immortality), the nectar of God in Vedas. In India, drinking of cow’s urine has been practiced for thousands of years. In Sushruta Samhita, urine was considered as an antidote to poisons and other properties of cow’s urine have been mentioned as weight loss and to cure leprosy, cardiac and kidney problems, indigestion, stomach ache, edema etc ^[11]. Panchgavya components (urine, milk, ghee, curd and dung) possess medicinal properties and are used singly or in combination with drugs of herbal, animal or mineral origin ^[12]. and used against many diseases, even against those not curable by allopathic treatments ^[13]. Cow urine is also used along with herbs to treat various diseases like fever, epilepsy, anemia, abdominal pain, constipation etc by traditional healers. It has been observed that important forest dwelling cows secrete so many herbal compounds in urine which are of high medicinal values. In cows plant origin dietary organic and inorganic compounds effectively get absorbed in the rumen and digested by bacterial activity. But there are some compounds, which do not disturb by the microbial enzyme action and secreted in their natural form in cow urine ^[9]. After going through the various literatures on cow urine it was revealed that cow urine has different phytochemical properties. Therefore, current research study was planned for preliminary phytochemical analysis in fresh cow urine, photoactivated cow urine, cow urine distillate, photoactivated cow urine extract of *A. indica* leaves and cow urine distillate extract of *A. indica* leaves.

2. Material and Methods

2.1 Authentication and collection of plant material

The leaves of *A. indica* were identified and authenticated by a Botanist from the Department of Botany, Maharashtra Udaygiri Mahavidyalaya, Udgir in the year 2019.

2.2 Plant material

A. indica leaves were collected and washed under running tap water and rinsed with distilled water to remove dust and debris. The leaves were detached out from each other after wash, segregated and shade dried. The shade dried leaves were powdered using a dry mechanical grinder. The dried powder was passed through the mesh sieve to obtain the fine powder and was stored in air tight container for its use whenever required.

2.3 Collection of urine

The urine samples were collected from non-pregnant group of Deoni cows reared under standard managerial practices at ILFC (Instructional Livestock Farm Complex), COVAS, Udgir. Natural voiding early morning mid-stream urine was collected in sterile glass container then subjected to examination of urine.

2.4 Preparation of cow urine distillate and photoactivated urine

2.4.1 Preparation of cow urine distillate (CUD)

The CUD was prepared using cow urine distillation unit (M/s Dolphin Labware, Mumbai). The fresh cow urine samples were filtered through four-fold muslin cloth and the filtered cow urine samples were subjected to a distillation unit where the distillate can be obtained after 4 – 5 hours of distillation.

The cow urine distillate was collected in a sterile transparent glass bottle, labelled and stored at 4^o C for its use whenever required. The urine from non-pregnant cows under study was used for preparation of cow urine distillate (CUD).

2.4.2 Preparation of photoactivated urine (PhU)

The fresh cow urine was filtered through four-fold muslin cloth. The filtered cow urine was photoactivated by maintaining it in sunlight for 72 hr in transparent glass bottle. The photoactivated urine was filtered through muslin cloth to make it free from debris and precipitated material. The photoactivated urine was labelled and stored at 4^oC in amber colour glass bottles for further use. The urine from non-pregnant cows under study was used for preparation of photoactivated urine (PhU).

2.5 Preparation of different extracts of leaves of *A. indica*

2.5.1 Photoactivated urine (PhU) extract of *A. indica* (PUEAi)

The extract was prepared as per the method suggested by Rosenthaler (1930). The 50 gm of powdered leaves of *A. indica* were added with 250 ml of photoactivated urine kept at room temperature for maceration period of 48 hrs. After maceration the mixture in the flask was filtered through the muslin cloth. The filtrate so obtained was once again filtered through Whatman filter paper No. 1. The filtrate extract was concentrated, evaporated to dryness until semi-solid masses were obtained. The extract was stored in refrigerator. The urine from all pregnant and non-pregnant cows under study was used for preparation of photoactivated urine (PhU) extract of *A. indica* (PUEAi).

2.5.2 Cow urine distillate (CUD) extract of *A. indica* (UDEAi)

The extract was prepared as per the method suggested by Rosenthaler (1930). The 50 gm of powdered leaves of *A. indica* were added with 250 ml cow urine distillate (CUD) of kept at room temperature for maceration period of 48 hrs. After maceration the mixture in the flask was filtered through the muslin cloth. The filtrate so obtained was once again filtered through Whatman filter paper No. 1. The filtrate extract was concentrated, evaporated to dryness until semi-solid masses were obtained. The extract was stored in refrigerator. The urine from all pregnant and non-pregnant cows under study was used for preparation of cow urine distillate (CUD) extract of *A. indica* (UDEAi).

2.5.2.1 Different preparation of non-pregnant cow urine thus obtained are

1. Fresh cow urine
2. Photo activated cow urine
3. Cow urine distillate
4. Cow Urine Distillate extract of *A. indica*
5. Photoactivated Urine extract of *A. indica*

2.6 Phytochemical analysis

The preliminary qualitative phytochemical analysis of fresh cow urine, photoactivated urine, photoactivated urine extract of *A. indica*, cow urine distillate and cow urine distillate extract of *A. indica* was done to detect presence or absence of various phytoconstituents namely alkaloids, glycosides, proteins, reducing sugar, tannins, sterols, phenolic compounds, and saponins as per the method described by Rosenthaler (1930) ^[14].

1. Test for Alkaloids

A small amount of extract was taken in test tube and added with 5 ml of 1.5% HCl (v/v) and then filtered. A few drops of each of the following reagents were added to the filtrate and mixed well, appearance of turbidity or any changes in colour to the test indicates the presence of alkaloids.

Dragendroff's reagent test:

The extract/filtrate was sprayed on filter paper using sprayer and was air dried. The Dragendroff's reagent was applied on prepared filter paper, development of orange to red color indicates presence of alkaloid.

Wagner's reagent test:

The little amount of extract/filtrate was added to Wagner's reagent, appearance of brown to flocculent precipitation revealed the presence of alkaloid

2. Test for Glycoside:**Benedict's reagent test**

Equal quantity of both the extract and benedicts reagent was added and heated to boil for two minutes, appearance of brownish to red colour indicate presence of glycoside.

Folin Wu copper reagent test

A few drops of Folin Wu copper reagent was added to little amount of extract, appearance of red color gives positive reaction for glycoside.

3. Test for Proteins:**Xanthoprotein test**

A small amount of the extract was added with 0.5 ml of concentrated HNO₃, appearance of white or yellow precipitate indicates the presence of proteins.

Biuret test

Few amounts of the extracts were added to 4% sodium hydroxide solution followed by a drop of 1% copper sulphate solution, the development of violet to pink colour indicates presence of proteins.

4. Test for Reducing Sugar**Benedict's reagent test**

The extract was added with benedicts reagent in equal amount

and mixture was heated for 2 minutes, appearance of brown to red colour indicates presence of reducing sugar.

Folin Wu copper reagent test

Few quantities of the extract were added with few drops of Folin Wu copper reagent, the development of red colour indicates presence of reducing sugar.

5. Test for Tannins:

A little amount of extract was added with few quantities of distilled water and the mixture was boiled and then filtered. The filtrate was used to carry out the following tests.

Lead acetate test

Few drops of 5% lead acetate solution were added to the filtrate, formation of precipitation indicates the presence of tannins.

Ferric chloride test

Few drops of ferric chloride were added to the little amount of the filtrate, development of green color revealed presence of tannins.

1. Test for Sterols**Salkowski reaction**

A small amount of extract was added with 2 ml of concentrated H₂SO₄ and was shaken for few minutes and mixed well, the development of red or brown colour indicates the presence of sterols.

7. Test for Phenolic Compounds

A small amount of extract was treated with 2 ml of ferric chloride solution and shaken for few minutes. The appearance of pale brown colour indicates presence of phenolic compounds.

8. Test for Saponins**Foam test**

A small amount of extract was treated with 2ml of sodium bicarbonate and added with distilled water, the mixture shaken vigorously. The development of froth to the test indicates presence of saponins.

3. Results and Discussion

Table 1: Phytochemical analysis of different extracts of *Azadirachta indica* leaves and cow urine solvents of non-pregnant cow.

Sr No.	Phytochemical	Tests	FUNPC	UDNPC	UDEAi	PUNPC	PUEAi
1.	Alkaloids	Dragendroff's	+ve	+ve	+ve	+ve	+ve
		Wagner's	+ve	+ve	+ve	+ve	+ve
2.	Glycosides	Benedict's	-ve	-ve	-ve	-ve	-ve
		Folin Wu's	-ve	-ve	-ve	-ve	-ve
3.	Tannins	Lead acetate	-ve	-ve	+ve	-ve	-ve
		Ferric chloride	-ve	-ve	+ve	-ve	-ve
4.	Proteins	Xanthoprotein	-ve	-ve	-ve	-ve	-ve
		Biuret	-ve	-ve	-ve	-ve	-ve
5.	Reducing Sugars	Benedict's	-ve	-ve	-ve	-ve	-ve
		Folin Wu's	-ve	-ve	-ve	-ve	-ve
6.	Phytosterols	Salkowski	-ve	-ve	+ve	+ve	+ve
7.	Phenolic Compounds	FeCl ₃ Solution	+ve	-ve	-ve	+ve	-ve
8.	Saponins	Foam	+ve	+ve	+ve	+ve	+ve

Note: '+ve' indicate Positive, '-ve' indicate Negative.

Abbreviations: 1) FUNPC: Fresh urine of non-pregnant cows 2) UDNPC: Urine distillate of non-pregnant cow 3) PUNPC: Photoactivated urine of non-pregnant cow 4) UDEAi: Urine distillate extract of *Azadirachta indica* 5) PUEAi:

Photoactivated urine extract of *Azadirachta indica* 5) A. *indica*: *Azadirachta indica* 6) AMA: Antimicrobial agents 7) CUD: Cow Urine Distillate 8) PhU: Photoactivated urine

The results of qualitative phytochemical analysis of *Azadirachta indica* leaves extracts is summarised in table-1.

The results showed that alkaloids and saponins were detected in solvents of non-pregnant cow urine *A. indica* leaves extracts. Tannins were detected in Cow urine distillate extract. Proteins were detected in Photoactivated urine and photoactivated cow urine extract of *A. indica*. Phytosterols were detected in cow urine distillate, photoactivated cow urine and photoactivated cow urine extract of *A. indica*. Phenolic compounds were detected in fresh cow urine of non-pregnant cow and photoactivated cow urine extract of *A. indica*. Glycosides and reducing sugar were absent in all cow urine extracts.

Alkaloids are organic nitrogenous substances (alkaline in nature) having remarkable physiologic and pharmacologic properties like stimulant, spasmolytic, vasodilator, anti-asthmatic, anti-arrhythmic etc. [15]. The presence of alkaloids represents the possibility of some biological activity of the extracts of *A. indica* such as anti-cholinergic, anti-tumour, anti-hypertensive, cough expectorant, anaesthetic, analgesic, muscle relaxant, anti-pyretic, anti-malarial [16]. The most important of these substances include alkaloids, tannins for cell growth, replacement and body building. Plant extracts are potential sources of novel therapies [17].

Saponins consist of polycyclic aglycones attached to one or more sugar side chains. Saponins have many health benefits such as the beneficial effects on blood cholesterol levels, cancer, bone health and stimulation of the immune system. Saponins show anti-fungal, antibacterial and anti-protozoal effects [15].

The presence of tannins represents the possibility of some biological activity of the extracts of *A. indica* such as anti-diarrheal, haemostatic, anti-hemorrhoidal, anti-inflammatory, astringent, and anti-infective. It can be used for immediate relief of sore throats, diarrhoea, dysentery, haemorrhaging, fatigue, skin ulcers and as a cicatrizing on gangrenous wounds. Tannins can also be used against poisons. It also possesses antioxidant effects. All these effects may be due to the presence of tannins in the extract of *A. indica* because tannins are previously reported to show such effects: [16]. Presence and absence of the phytochemical constituents depend on the test applied for the qualitative detection of secondary metabolites.

Phenolic compounds in *A. indica* leaves extracts have been reported to be associated with anti-oxidative action which provides protection against free radicals that damage cells and tissues [15]. For instance, vitamins A, C, E, and phenolic compounds such as flavonoids and tannins found in plants, all act as antioxidants. Flavonoids have been shown to work as Plants anti-tumour (benign, melanoma) agents involving a free-radicals quenching mechanism (i.e., OH, ROO). In fact, many studies have shown that flavonoids play significant multiple roles including mutagenic, cell damage, and carcinogenic, due to their acceleration of different aging factors. [18].

4. Conclusion

Neem has long history as a medical plant with diverse therapeutic applications. The phytochemical experiments performed during the current study confirms that the plant extracts are rich in some phytochemicals like alkaloids, tannins, phytosterols, phenolic compounds and saponins. Cow urine is traditionally used by many people as an alternative treatment for a variety of health ailments capable of curing blood pressure, blockage in arteries, arthritis, diabetes, heart

attack, cancer, thyroid, asthma, psoriasis, eczema, prostrate, fits, AIDS, piles, migraine, ulcer, acidity, constipation, gynaecological problems, ear and nose problems and several other diseases. These findings support the traditional knowledge of local users and sustainable use of such plant resources along with cow urine. Study suggested a number of active constituents might be present in the neem and in non-pregnant cow to control pathogens. So, the pharmacological activity of neem-cow urine extract is due to the presence of different compounds with varying complexity.

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