# A comparative analysis of the antimicrobial activities of *Andrographis paniculata* (Burm. f.) Wall. ex Nees plant extracts and its pure diterpenoids: Andrographolide and neoandrographolide

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***Abstract***
*The current study assesses the comparative potency of Andrographis paniculata crude extracts and their pure bioactive compounds against gram-positive, gram-negative and fungal pathogens. This study also estimated the polyphenolic contents present in different solvent extracts. A total of four solvent extracts of different polarities isolated from Andrographis paniculata and its major bioactive compounds were investigated against human pathogenic microbes. The antibacterial activities and minimum inhibitory concentrations were assessed by the broth dilution assay. The total phenolic and flavonoid contents were estimated by using the Folin–Ciocalteu reagent method and Aluminum chloride colorimetric method, respectively.*

*Andrographolide and neoandrographolide both have significant antibacterial activities against all bacteria, but are completely inactive against Candida albicans. Ethanolic and ethyl acetate extracts demonstrated antimicrobial activity against each microbe investigated in this study. Both diterpenoids and ethanolic extract were effective against Staphylococcus aureus. This study tried to conclude that most of the solvent extracts contain anti bacterial activity, but andrographlide and neoandrographolide are not the only antibacterial agents present in the crude of Andrographis paniculata and there are also other phytochemicals present which have great activity as an antifungal agent.*

**Keywords:** Andrographis paniculata andrographolide, neoandrographolide, antibacterial, antimicrobial, herbal, phytochemicals, diterpenoid.

## Introduction

Since ancient times, herbs and their natural compounds have been used as a basis for the development of new drugs[12]. According to the WHO, 80 percent of the world's population trusts traditional plant-based systems since they are relatively safe, inexpensive and provide significant therapeutic advantages.[20] India is one of the few countries that has a wealth of medicinal plants as well as traditional knowledge on how to use herbal medicines to treat a variety of diseases[13].

We evaluated the ethnomedicinal knowledge of the antibacterial activity of numerous plants and decided on *Andrographis paniculata* (AP) (Burm.f) wall Ex.nees. (Acanthaceae) which is known as the "King of Bitters".

According to the literature, AP includes a variety of bioactive phytoconstituents. The first and most significant compounds are ent-labdane type diterpenoids (Figure 1)[18]. Andrographolide (Andro), neoandrographolide (Neo) and their diterpenoid derivatives are well-known and prime bioactive chemical constituents of AP[7]. A P have been used in China, India and Southeast Asia for the therapeutic treatment of viral and bacterial infections since ancient times. Its constituents have been the subject of intense phytochemical research since 1911[25]. Both andro and neo are present in major quantities in the alcoholic extract as its polarity is as high as alcohol[5,14].

In this study, we compared the antibacterial and antifungal activities of different solvent extracts of AP and its pure phytochemicals. This study tried to assess the bioactivity of Andro and Neo against gram positive-bacteria, gram-negative bacteria and fungus strains. We also assessed the percent yield, qualitative and quantitative analysis of the extract from the plant.

## Material and Methods

**Reagents and biomolecules:** SRL Ltd. provided analytical quality extraction solvents such as ethanol, ethyl acetate and hexene. For total phenolic content analysis, Sigma Aldrich provided aluminum chloride, potassium acetate, Folin-Ciocalteu reagent and gallic acid; for total flavonoid content analysis, Sigmaaldrich provided rutin, aluminum chloride and potassium acetate.

Andrographolide and neoandrographolide were purchased from Sigma-Aldrich, Himedia, India, provided bacterial culture reagents: Muller-Hinton agar, Luria broth and Sabouraud dextrose agar.

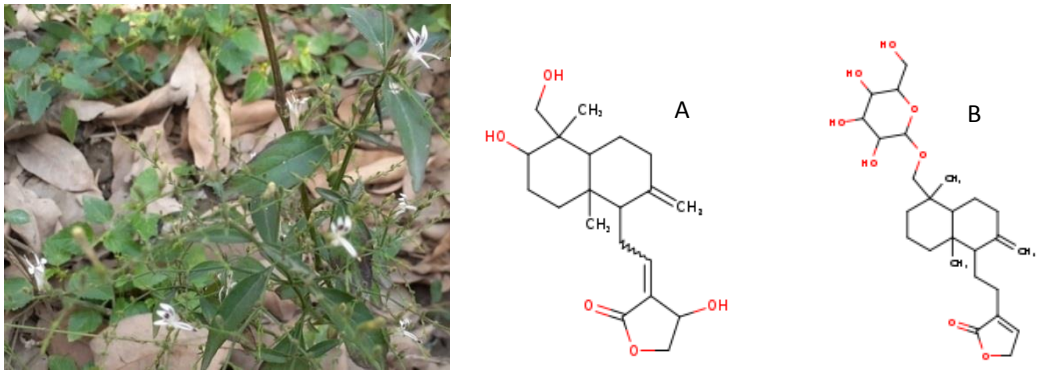


**Fig. 1: Morphology of *Andrographis paniculata.* Chemical structure of (A) Andrographolide and (B) Neoandrographolide**

**Plant material & sequential extraction method:** Leaves of around 0.3-0.5 meter tall AP herbs were obtained from the garden of Banaras Hindu University's Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Varanasi, India (25.272 N, 82.998 E). After authentication, a voucher of the specimen was placed in the herbarium of the Faculty of Ayurveda, IMS, BHU, Varanasi (DG/21-22/354). The leaves were thoroughly rinsed, dried in a shaded place at room temperature and then pulverized with a mixer grinder. For 32 hours, 100 g of powdered material was used in the Soxhlet extractor with 300 ml of hexane as the solvent. With the help of a rotatory evaporator, the light green extract was filtered and evaporated to dry at 45ºC. The same sample powder was allowed to dry before being extracted with ethyl acetate in a Soxhlet extractor. The same procedure was carried out with ethanol and then water. For future usage, all four extracts were kept in a sealed container at -40°C[21].

**Qualitative phytochemical analysis:** Standard test methods were used for qualitative phytochemical analysis i.e. amino acids, glycosides, flavonoids, tannins, alkaloids, saponins and phenolic compounds. The following tests were used: Mayer's test for amino acids, for carbohydrates Fehling's test, for flavonoids Shinoda's test, for proteins, Biuret's test, for phenolics, ferric chloride test, for saponins, honeycomb formation test, for tannins, ferric chloride test and for phytosterols, Libermann-Buchard's test were applied[23]. This analysis was performed in the department of Dravyaguna, Faculty of Ayurveda, IMS, BHU, Varanasi.

**Quantitative phytochemical analysis**

**Determination of total phenol content:** TPC was determined using the Folin–Ciocalteu reagent method with some modifications[17]. To make a final volume of 3 ml of the reaction mixture, 0.1 ml of 1 mg/ml extract in distilled water, 0.2 ml of FC reagent mixed in a test tube and allowed to stand for 5 minutes, 2 ml of aqueous sodium carbonate solution (7%) and 0.7 ml of distilled water were added. After 15 minutes of incubation at room temperature, absorbance was recorded at 750 nm by using a spectrophotometer. All tests were done in triplicate. Various concentrations of gallic acid were used to generate the standard curve. Total phenol values were expressed as milligrams per gram of gallic acid equivalents. The assay was performed in the Department of Botany, MMV, BHU, Varanasi.

**Determination of total flavonoid content:** Total flavonoid content was determined by using the aluminum chloride colorimetric method[3,] Estimation of total flavonoid content in AP by two complementary colorimetric method with slight modification. 0.1 ml of 1 mg/ml extract in ethanol was mixed with 0.1 ml (2%) aluminium chloride, 0.1 ml (1 M) potassium acetate and 2.7 ml of ethanol to make a final volume of 3 ml of the reaction mixture. After 30 minutes of incubation at room temperature, absorbance was recorded at 415nm. Each test was done in triplicate. TFC was estimated using rutin as a standard and expressed as milligrams per gram of rutin equivalents.

**Antimycobacterial assay:** A total of two Gram-negative bacteria, *E. coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC 17978), Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and a fungal isolate, *Candida albicans* (ATCC 90028), were employed in the investigation for antimicrobial assay using broth dilution assay. According to CLSI guidelines document M27-A3, microbes were cultured on their respective nutrient media, which are Mueller-Hinton agar (MHA) for bacterial growth and Sabouraud's dextrose agar for fungal growth[4]. A pure culture colony was suspended in saline to adjust the turbidity according to the MacFarland Scale of 0.5 ($10^6$ CFU/ml).

This suspension was used as a standard inoculum. The working stock of the test compound was prepared at 5 mg/ml. In the walls of the microtiter plate, equal volumes of test compounds in various dilutions (2.5, 1.25, 0.625, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01 mg/ml) and nutrient agar were mixed. All wells were subjected to two-fold serial dilution to determine the MIC. Approximately $5x10^6$ CFU/ml of 0.1 ml of bacterial culture was added to each well of the plate. The inoculated microtiter plate was incubated at 37ºC for 24 hours. At the end of the incubation, the lowest concentration of sample containing culture well producing no visible growth of bacteria (no turbidity) when compared to the control well was considered the Minimum Inhibitory Concentration against the respective microbe.

## Results

**Extractive yield:** The extractive yield of AP is diverse among different solvents. The highest yield was found in the aqueous extract, followed by other solvent extracts. The extractive yields of all extracts can be ranked from highest to lowest in the following sequence; aqueous (17.21± 0.8) > ethyl alcohol (15.54±0.5) > hexene (6.12± 0.32) > ethyl acetate (0.62±0.07).

**Total Phenolics and Flavonoid contents:** The result shows that all solvent extracts have a TPC range from 7.9±0.11 mg/ml to 23.33±0.47 mg/ml Gallic acid equivalent (GAE) and a TFC range from 58.77±0.9 mg/ml to 282.11±34 mg/ml Rutin equivalent (RE). TPC and TFC were found in significant amounts in hexane and ethanolic extracts, respectively.

**Anti- Microbial activity:** All four different polarity solvent extracts and both active phytoconstituents were subjected to *S. aureus, A. bumannii, E.coli* and *C. albicans* (Table 2). The MIC range of solvent extracts is 0.31 mg/ml to 2.5 mg/ml, whereas pure AP compounds range from 0.075 mg/ml to 1.25 mg/ml. Ethyl acetate extract andro and neo contain greater activity against *S. aureus* with 0.31 mg/ml, 0.075 mg/ml and 0.15 MIC respectively. Neoandrographolide has better activity on *A. bumannii* (0.62 mg/ml) than other test compounds. Ethanolic extract was more active against *E. coli* than other test compounds while ethyl acetate extract was more active against fungi (0.31 mg/ml), though both diterpenoids were inactive against *C. albicans*.

**Table 1**
**Extractive yield, total phenol and flavonoid content of different solvent extracts of *Andrographis paniculate***

| Solvent/Extracts | % Yield (w/w) | TPC | TFC |
|---|---|---|---|
| | | mg/g of standard equivalents | |
| Hx/AP | 6.12± 0.32 | 7.9±0.11 | 282.11±34 |
| EA/AP | 0.62±0.07 | 9.23±0.2 | 113.7±10 |
| EtoH/AP | 15.54±0.5 | 23.33±0.47 | 59.3±1.6 |
| $H_2O$/AP | 17.21± 0.8 | 17.93± 2.8 | 58.77±0.9 |

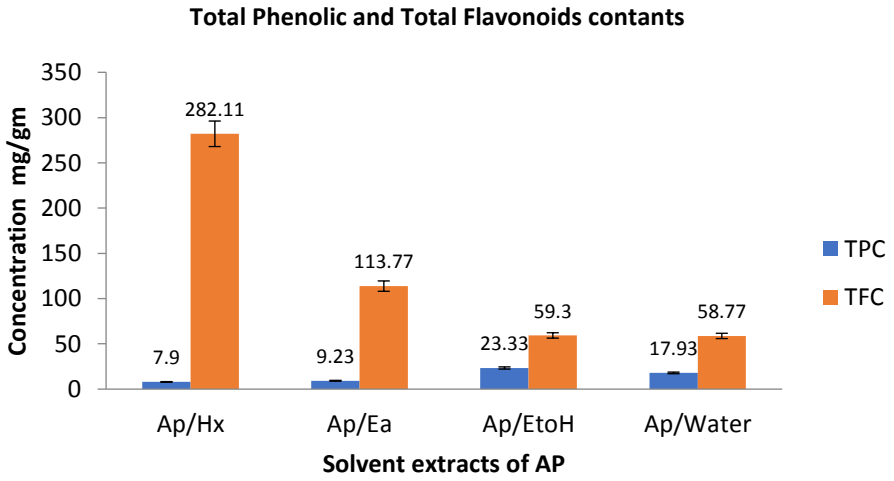


**Fig. 2: Comparative concentrations of polyphenolic contents present in different solvent extracts.**
**Ap: *andrographis paniculata*, Hx: hexane, Ea: ethyl acetate, EtOH: ethanol**

**Table 2**
**Antimicrobial activity of different solvent extracts of *andrographis paniculata* and comparison with pure Andrographolide and Neoandrographolide compounds.**

| Microorganism | Minimum Inhibitory Concentration (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Plant Extract | | | | Phytochemicals | | |
| | Hexane | Ethyl Acetate | Ethanol | Water | Andrographolide | Neoandrographolide | Positive drug control |
| *S. aureus* | 1.25 | 0.312 | 0.625 | 2.5 | 0.075 | 0.15 | < 0.009 [a] |
| *A. baumannii* | NA | 2.5 | 1.25 | NA | 1.25 | 0.62 | < 0.006 [b] |
| *E. coli* | NA | 2.5 | 1.25 | NA | 1.25 | 1.25 | < 0.004 [a] |
| *C. Albicans* | 1.25 | 0.312 | 0.625 | 1.25 | NA | NA | < 0.002 [c] |

Streptomycin (a), Ampicillin(b), Fluconazole (c), no activity (NA)

## Discussion

Herbal medicines are typically the only widely available kind of medicine in developing countries, but still their access to hospitals and health care is restricted. Herbal medicines are found to be non-toxic, have fewer adverse effects, have a wide range of biological activities, are culturally acceptable and are readily available at low prices. Further their chemical constituents are thought to be more suitable for the human body than synthetic medications since they are a part of the physiological functions of living flora[9,11].

According to the World Health Organization, herbal medicines are used two to three times more than conventional pharmaceuticals across the world[20.] AP is used as a herbal medicinal plant due to its wide range of pharmacological properties. AP and its constituents have been observed to have antimicrobial potency against various microbes widely. In our study the gram-positive bacteria *S. aureus* and the fungus *C. albicans* were the most vulnerable towards the plant extract. However, ethyl acetate extract was observed to be the most active sample against *S. aureus*. Hexane and aqueous extracts of AP did not show any activity against *A. baumannii* and *E. coli* bacteria.

Whereas andrographolide and nooandrographolide were always at the pivot of research in many previous studies. In this study andro and neo both have activity against gram positive and negative bacteria, but are inactive against fungus. Although both diterpenoids are present in polar solvent extracts, there might be other compounds that are active against *C. albicans*. In spite of that, both andro and neo have a vast range of medicinal properties[8.] A study by Singha et al. found a wide range of concentrations of crude powder of A. paniculata to have antibacterial activity against *Salmonella, E. coli, Shigella*, gram-A streptococci and *Staphylococcus aureus* in vitro[16]. A similar outcome was found in a crude aqueous extract of AP that exhibited antimicrobial activity against *S. aureus*, methicillinresistant *S. aureus and Pseudomonas aeruginosa*[24]. Ethanolic extract was found active against enterohemorrhagic strains of *E. coli.*[19]

In another study, AP was reported to have significant antibacterial activity against nine bacteria: *Salmonella typhimurium, E. coli, Shigella sonnei, Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumonia, Streptococcus pyogenes, Legionella pneumophila* and *Bordetella pertussis*[22]. A study by A. Prabu et al. reported that alcoholic extract of AP had better activity (250 µg/ml) against sensitive and MDR strains of *Mycobacterium tuberculosis*[13].

This was confirmed in a study on andrographolide where MIC was found sensitive at 100 µg/ml against bovine tuberculosis[15]. Polyphenols are found to be a potent antioxidants. In a study andrographolide demonstrated significant activity against *S. aureus, E. coli* and *A. baumannii* with MICs of > 0.1 mg/ml, > 1.0 mg/ml and > 1.0 mg/ml, respectively. However, in a study andrographolide was found to be inactive against C. albicans[1].

According to our results of the polyphenolic determination assay, hexane extract had a significant amount of total flavonoids and methenolic extract contains more phenolic content than other extracts. A previous study by Kaneria et al[10] also showed agreement in flavonoids estimation like our study. The percent yield of extracts in our study also follows a similar pattern like their study study.

The medicinal plants are an important source of antibacterial and antifungal compounds with substantial activity against pathogenic microorganisms and they provide a novel source of antibacterial and antifungal agents with significant activity against pathogenic microorganisms. Plant extracts showed greater antibacterial than antifungal activity in our study. Gram-positive bacteria were more sensitive towards the all test samples than Gram-negative bacteria. This also confirmed the fact that Gram-positive bacteria are more vulnerable to herbal drugs than Gram-negative bacteria[2].

## Conclusion

The present investigation reveals the biological value of the phytoconstituents present in AP. Although the majority of phenol and flavonoid content are present in hexane and ethanolic extracts of AP and the major antimicrobial activity is also present in ethanolic extracts andrographolide and neo andrographolide were restricted to bacterial pathogens. There is other active phytoconstituent that act against fungal pathogen. Gram-positive bacteria are more susceptible to both diterpenoids than gram-negative bacteria and ethyl acetate and ethanolic extract have significant susceptibility against *candida albicans*. The promising results of this study might lead to the development of a potent antibiotic against pathogens. It also encourages young researchers to investigate the other bioactive constituents of *Andrographis paniculata*.

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(Received 30th October 2021, revised 29th May 2023, accepted 25th June 2023)