

Antimicrobial Activity of Chemical Compounds from *in vivo* Roots and *in vitro* Callus of *Withania somnifera* (L.) Dunal

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Abstract *Withania somnifera* is an important medicinal plant and a major source of alkaloids and steroids. Large callus was obtained from stem explants in MS medium with additives of 0.5 mgL⁻¹ 6-Benzylaminopurine (BAP) + 1.5 mgL⁻¹ α -Naphthalene acetic acid (NAA). In phytochemical screening of *in vivo* and *in vitro* materials of *W. somnifera*; reducing compounds, cardiac glycosides, basic alkaloids, carotenoids, volatile oils and saponins were detected. Phytochemical compounds found in callus of *W. somnifera* stopped the growth of bacteria *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Klebsicela pneumonia* more effectively than the compounds found in its natural root. Whereas active phytochemical compound found in natural root was more effective to stop the growth of bacteria *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Salmonella typhimurium*. From this study it can be predicted that specific compounds which is not found in roots of naturally growing plants plant be found in the callus of *W. somnifera*.

Keywords: *in vivo*, *in vitro*, *Withania somnifera*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsicela pneumonia*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhimurium*

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1. Introduction

Withania somnifera is a popular medicinal plant known as Ashwagandha. Medicinal plants have served as a common link between the traditional and modern science as they are the main source of medicaments involved through the centuries [1]. Medicinal plants represent a rich source of antimicrobial agents [2]. In the traditional medicine system, this plant is claimed to have potent aphrodisiac, memory related problems, insomnia, skin problems, increase energy, strength, increase vital fluids, blood, lymph, semen and cell production [3].

The secondary metabolites are known as natural products that include broad categories of compounds such as alkaloids, steroids, terpenoids, phenols etc. Although secondary metabolites were first recognized in 1873 [4], their function was known only in 1888 [5]. It is believed that more than 1, 00,000 different structures of secondary metabolites can be synthesized by organisms to an extent of 10⁹ tons per year [6]. Chemical constituents of *Withania somnifera* are alkaloids (ashwagandhine, cuscohygrine, anahygrine, tropine etc), steroidal compounds including ergostane type steroidallactones, withaferin A, withanolides A-y, withasomniferin-A, withasomidienone, withasomniferols A-C, withanone etc [7].

Two important aspects of *in vitro* culture in medicinal plants are micropropagation / for conservation / mass

propagation and *in vitro* production of phytochemicals to improve the production of pharmaceuticals such as alkaloids, terpenoids, steroids, saponins, phenolics, flavanoids, food additives, amino acid etc.

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by the pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity [8,9]. The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to search new infection fighting strategies [10]. Thus, the development of new antibiotics is becoming a global challenge for the preoccupying research institutions, pharmaceutical companies and academic institutions [11]. However, the past record of rapid and widespread emergence of resistance to newly introduced antibiotics indicates that, even new antibiotics are expected to have a short life [12]. In this regards, the exploration of new antibacterial agent would be an urgent need. Thus in this research we have undertaken the antimicrobial activities of chemical compounds obtained from naturally grown plants and callus of *W. somnifera*.

2. Material and Methods

2.1. Callus Culture

The nutrient media used for present study was Murashige and Skoog 1962 (MS) media. It is the basal medium augmented with different concentrations of auxins and cytokinetins. The P^H of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl. The medium was solidified with 8.0 gL⁻¹ agar-agar. The culture tubes containing medium were sterilized by autoclaving at pressure of 15 psi and temperature of 121°C for 20 minutes.

For inoculation of explants, the laminar airflow chamber was thoroughly cleaned by spirit. All the necessary material such as glassware, metal instruments were autoclaved. Culture tube with media, sterile water, rubber bands, aluminum foils, match box, marker pen etc were kept in the laminar airflow under ultraviolet (UV) light irradiation to ensure sterile condition. Then, explants inoculated aseptically in the laminar airflow cabinet.

The different explants *i.e.* shoot tips, nodes, leaves and roots were taken from aseptically grown seedlings of eight to ten weeks old under *in vitro* condition. These explants were inoculated on MS basal medium supplemented with different concentrations and combinations of 6-Benzylaminopurine (BAP) and α -Naphthalene acetic acid (NAA). Callus was obtained from the node, shoot tip and leaf, which was further sub-cultured on MS basal medium augmented with different concentration of NAA and kinetin.

2.2. Phytochemical Screening:

Powdered plant materials, dried roots from of *in vivo* grown plants and *in vitro* developed callus of *W. somnifera* were used for phytochemical screening test. Chemical tests were carried out on the aqueous and alcoholic extracts using standard procedures to provide by Edeoga 2005 and Salehi Surmagi *et al.* 1992 [13,14].

a. Test for coumarins: The ether extract (4 ml) was concentrated to yield a residue, which was dissolved in hot water (4 ml). After cooling, the solution was divided into two test tubes. The first test tube was used as a control. To the second test tube 10 percentage of ammonium hydroxide solution was added drop by drop until pH 8 and was then observed under UV light. Greenish yellow fluorescence was not observed indicating the absence of coumarins.

b. Test for alkaloids: About 2.5 gm of sample was extracted with 10 mL methanol and evaporated to dryness and the residue was heated on a boiling water bath with 2N HCl (5 ml). The resulting mixture was centrifuged for 10 minute at 3000 rpm to remove residue. First 1 ml of the filtrate was treated with a few drops of Mayer's reagent and the second 1 ml portion was treated with equal amounts of Wagner's reagent. The samples were then observed for presence of precipitation.

c. Test for carotenoids: The petroleum extract was concentrated and then treated with conc. sulfuric acid (1 ml). No orange yellow color similar to the extract solution was developed which on long standing did not turned red indicating the absence of carotenoids.

d. Test for cardiac glycosides (Keller-Killani test): 5 ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride

solution. This was under layer with 1 ml of concentrated sulfuric acid. A brown ring of the interface indicate deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

e. Test for saponins: About 2.5 gm of the plant material was extracted with 10 ml of boiling water. After cooling, the extract was shaken vigorously to froth and was then allowed to stand for 15-20 min and classified for saponins content as follows: no froth-negative; froth less than 1 cm-weakly positive; froth 1.2 cm high-positive; and froth greater than 2 cm-strongly positive.

f. Test for tannins: About 0.5 gm of sample was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride (FeCl₃) solution was added and observed for brownish green or blue black coloration. A blue black precipitate was taken as evidence for the presence of tannins.

g. Test for terpenoids (Salkowski Test): 5 ml methanol extract, corresponding to 2.5 gm of plant material, was mixed in 2 ml chloroform, and concentrated H₂SO₄ (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids.

h. Test for polyphenols (ferric chlorids test): The methanolic extract (1 ml) was mixed with water (1 ml). To this solution, 1% ferric chloride solution (3 drops) was added. A greenish blue color was not developed indicating the absence of polyphenols.

i. Test for reducing compounds (Fehling test): The methanolic extract (1 ml) was diluted with water (1 ml). To this solution, Fehlings reagent 1/1 mixture of fehlings reagent A and B (1 ml) was added and then the mixture was warmed over a water bath for 30 minutes. A brick red produced indicating the presences of reducing compounds.

j. Test for phlobatannins: Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

k. Test for flavonoids: 5 ml of dilute ammonia solution was added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H₂SO₄. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colorations disappeared on standing. Few drops of 1% aluminum solution were added to a portion of each filtrate. A yellow colouration was observed indicating the presence of flavonoids. A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed indicating a positive test for flavonoids.

l. Test for coumarin derivatives: The ether extract solution (4 ml) was concentrated to yield a residue, which was dissolved in hot water (4 ml). After cooling, the solution was divided into two test tubes. The first test tube was used as a control, to the second test tube, 10% ammonium hydroxide solutions was added drop

by drop until pH 8 and was then observed under UV light. Yellow fluorescence in second test tube was observed indicating the presence of coumarin derivatives.

m. Test for volatile oils (Spot test): The Petroleum extract (4 ml) was concentrated to yield a residue. To this residue methanol (1 ml) was added and shaken vigorously then filtered. Few drops of the filtrate were spotted on a filter paper. A yellow spot was persistent even after evaporation indicating the absence of volatile oils.

2.3. Antibacterial Screening

Inhibition of bacterial growth was tested by using the paper disc diffusion method [15,16] with some modifications.

2.3.1. Preparation of Extract

Two grams of air dried callus was soaked in 25 ml of methanol for 24 hours and filtered using standard filter paper (Whatman no. 1). The residue was soaked again with 25 ml fresh methanol and filtered after 24 hours. Same process was repeated once again. The extract after treating with 75 ml (25 ml x 3 times) methanol was then filtered. The filtrate was transferred into beakers and allowed to evaporate until completely dry; the extract was re-suspended in 2 ml of methanol. The concentration of the final extract was 1g material /1ml.

2.3.2. Collection of Test Organisms

Multi drug resistance bacteria are obtained from Central Department of Microbiology, Tribhuvan University. Three gram-positive (*Bacillus subtilis*, *Klebsiella pneumoniae* and *Staphylococcus aureus*) and four gram-negative (*Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*). Bacterial strains were taken on slants and later cultured on petri plates in nutrient agar media.

2.3.3. Preparation of the Test Discs

Sterile test discs were prepared by dipping and saturating sterile filter paper discs (6 mm diameter) in plant extract. For negative control methanol paper discs were used, prepared by dipping the disc into the methanol, while tetracycline paper discs were used as positive control. For tetracycline paper discs 10 ml solution was prepared mixing 0.8 ml tetracycline solution (prepared by dissolving 500 mg tablets of tetracycline in 20 ml methanol) with 9.2 ml of methanol. The final concentration of tetracycline was 0.25 mg/ml.

2.3.4. Preparation of Culture Media

I. Nutrient Agar: Nutrient agar was prepared with the help of manufactures (Hi-media) recommendations. 28 g of nutrient agar was weighed and dissolved in distilled water to make final volume of 1000 ml. It was sterilized by autoclaving the media inside the round bottomed flask at 15 lb pressure and 121°C for 15 minutes. It was then cooled to 50°C. About 20 ml of media was poured to sterile petri plates aseptically and labeled properly. For the slant preparation, the required amount of media was poured in appropriate sized screw capped bottle, autoclaved and cooled in tilted position to make slant.

II. Nutrient Broth: Nutrient broth was also prepared with the help of manufactures (Hi-media) recommendations. 13 g of powder was weighed and dissolved in distilled water to make final volume of 1000 ml. It was sterilized by autoclaving at 15 lb pressure and 121°C for 15 minutes inside the conical flask. It was cooled and 10 ml of it was poured inside the suitable sized screw capped bottle and again sterilized.

2.3.5. Preparation of Standard Culture Inoculums

Three to five colonies of similar appearance of the organism to be tested were aseptically isolated with the help of inoculating loop from primary culture plate. It was transferred to a tube containing 10 ml sterile liquid media of nutrient broth. The tube was incubated overnight inside the incubator at 37°C.

2.3.6. Transfer of Bacteria on Petri Plates

The agar plates for the assay were prepared by labeling them with the date, the name of bacteria and the name code of the discs. The inoculums of bacteria were transferred into petri dish containing solid nutrient media of agar using sterile swab. The sterile cotton swab was dipped into a well mixed saline test culture and removed excess inoculums by pressing the saturated swab against the inner wall of the culture tube. The swab was used to spread the bacteria on the media in a confluent lawn. It was done by rotating the petri plates at 90° and continuing the spread of bacteria. One swab was used for one species of bacteria. The culture plates were allowed to dry for five minutes.

2.3.7. Placing Test Discs

Dried test discs were transferred on bacterial lawn under aseptic conditions using flame-sterilized forceps each time. Each disc was placed gently on the agar surface on equidistance and patted with the forceps to ensure the disc adhere to the surface of agar. The petri plates were incubated in an inverted position for 24 hours at 37°C.

3. Result and Discussion

3.1. Callus Culture

Different explants of *W. somnifera* were cultured on MS basal medium supplemented with NAA, BAP, 2, 4-D, and kinetin with various concentration and combination. Proliferation of callus was observed in all the conditions of MS media supplemented with various concentration and combination of BAP and NAA. The best growth of callus was observed in the MS medium supplemented with 0.5 mg L⁻¹ BAP + 1.5 mgL⁻¹ NAA at 8 weeks. The callus growth were observed on the MS medium supplemented with 1.0 mgL⁻¹ BAP + 1.0 mgL⁻¹ NAA, 0.5 mgL⁻¹ BAP + 0.5 mgL⁻¹ NAA, 1.0 mgL⁻¹ BAP + 1.5 mgL⁻¹ NAA, 1.5 mgL⁻¹ BAP + 1.5 mgL⁻¹ NAA and 0.5 mgL⁻¹ BAP + 2.0 mgL⁻¹ NAA were similar. MS basal medium in exogenous supply of 0.5 mgL⁻¹ BAP +1.5 mgL⁻¹ NAA was the best among all tested medium and observed large callus.

Maximum callusing (100%) was obtained from root and cotyledonary leaf segments grown on MS medium supplemented with a combination of 2 mgL⁻¹ (9.1 μM) 2,4-D and 0.2 mgL⁻¹ (0.9 μM) KN [17]. Vishnoi, *et al.*

1979 reported that callus formation has been induced in anthers of *Withania somnifera* cultured on Murashige and Skoog's (1962) medium supplemented with BAP (10^{-6} M) [18]. The callus cultures of *Withania somnifera* were initiated and maintained on MS media supplemented with Dicamba (2 mg/l), Kinetin (0.1 mg /l) and Sucrose (3% w/v) [29]. In this study, maximum callusing was obtained from stem segments on MS medium supplemented with a combination of 0.5 mg L^{-1} BAP + 1.5 mg L^{-1} NAA.

3.2. Phytochemical Screening of Callus (*In vitro*) and Roots from *In vivo* Grown Plant

In phytochemical screening of *W. somnifera* observed similar phytochemical compound of *in-vivo* and *in vitro* material. In extract of different solvents such as methanol, hexane, ethyl acetate, petroleum ether and water. Methanol extract gave the positive test of reducing compound and cardiac glycosides. Volatile oil was present in petroleum ether and ethyl acetate extraction. Basic alkaloids and carotenoids were found in ethyl acetate extraction. Water extraction gave positive test of saponins.

Table 1. Phytochemical screening test of *Withania somnifera*

S.N.	Family of natural constituents.	Name of test	Screening test	
			<i>in vivo</i> plant	<i>in vitro</i> callus
1	Polyphenols	Ferric chlorides test	-ve	-ve
2	Reducing compounds	Fehling's test	+ve	+ve
3	Tannins	Ferric chlorides test	-ve	-ve
4	Phlobatannins	hydrochloric acid test	-ve	-ve
5	Cardiac glycosides	Kedde's test	+ve	+ve
6	Terpenoids	Salkowski's test	-ve	-ve
7	Essential oil	Spot test	+ve	+ve
8	Flavonoids	Aluminum solution test	-ve	-ve
9	Saponins	Froth's test	+ve	+ve
10	Carotenoids	Sulphuric acid test	+ve	+ve
11	Basic alkaloid	Dragendorff's test Maeyer's test	+ve +ve	+ve +ve
12	Coumarins	UV method	-ve	-ve
13	Coumarins derivatives	UV method	-ve	-ve

3.3. Comparative Study of Compounds in Natural Plant roots (*in vivo*) and callus (*in vitro*)

In comparative study of phytochemical compound of *W. somnifera* in *in vitro* and *in vivo* materials was observed almost identical chemical constituent with the help of TLC. Most important bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds [20]. The antibacterial properties exhibited by extracts may be associated with presence of tannins, saponins, cardiac glycosides and alkaloids found in the plant extract [21,22,23]. Both the test material in the present study contain reducing compounds, cardiac glycosides, essential oil, saponins, carotenoids and basic alkaloid whereas

comparatively both plant material contain the similar group.

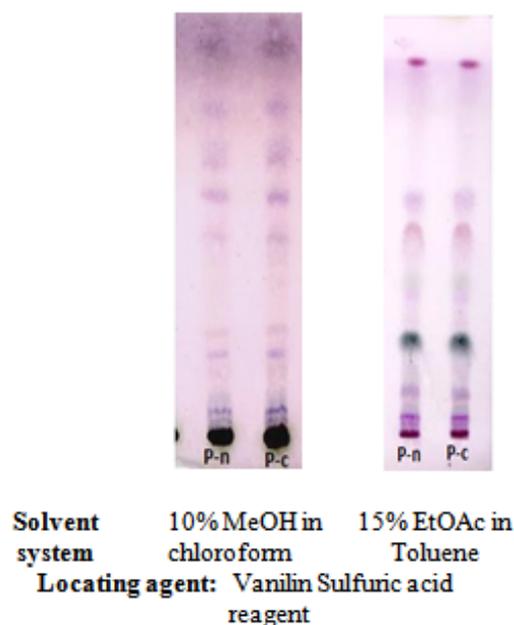


Figure 1. Co-TLC of MeOH extract of natural plant (P-n) and callus (P-c) [I], Co-TLC of Chloroform extract of natural plant and callus [II]

The water extract of *Withania somnifera* was found to contain steroids, terpenoids, flavonoids, phenol, quinones and catechin, ethanol extracts show steroids, tannins, phenol, quinines and methanol extracts exhibit only tannins, phenol and quinines [24]. Extracts of *Withania somnifera* presence of phenols, flavonoids, tannins, saponins, alkaloids, steroids, terpenoids, glycosides and reducing sugars which could account for its varied medicinal properties like anti-inflammatory, anti-spasmodic, anti-analgesic, neuroprotective and diuretic effects [25]. In this study, *in vitro* and *in vivo* material contains similar secondary metabolites compound. Secondary metabolites found in these plants used for the production of various pharmaceutical compounds. Thus, callus culture could be employed as a possible alternative to produce such industrial compounds. Tissue culture techniques provide continuous, reliable, and renewable source of valuable plant pharmaceuticals and can be used for the large scale culture of the plant cells from which these secondary metabolites can be extracted. Alkaloids are one of the most important secondary metabolites known to play a vital role in various pharmaceutical applications leading to an increased commercial importance in recent years.

MIC of the extract of natural root of the *Withania somnifera* inhibited and fully prevented the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhimurium* and *Klebsicela pneumonia* at of 5.0 mg/ml, *E. coli* and *Staphylococcus aureus* at a concentration of 1mg/ml, *Proteus vulgaris* at a concentration of 40mg/ml. But in the callus extract of *W. somnifera* ZOI was observed in *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Klebsicela pneumonia* at the concentration of 1mg/ml, *Escherichia coli* at the concentration of 5.0 mg/ml, *Staphylococcus aureus* at a concentration of 40 mg/ml, *Salmonella typhimurium* and *Bacillus subtilis* at the concentration of 60 mg/ml. Hence callus as well as natural root contains active phytocompounds.

Callus of *W. somnifera* contains more active phytochemical compounds which inhibited the growth of *Klebsicela pneumonia*, *Proteus vulgaris* and *Pseudomonas aeruginosa* than inhibited by natural root of *W. somnifera*. However natural root of *W. somnifera* contains the active phytochemical compounds against

Staphylococcus aureus, *Bacillus subtilis*, *Salmonella typhimurium* and *Escherichia coli* is highly effective than that of callus. Phytochemical screening and microbial activities of callus and natural root showed that these materials contain similar phytochemical compounds with different concentration.

Table 2. Comparative antibacterial activity of methanol extract of *in vitro* and *in vivo* materials

Concentration	<i>Pseudomonas aeruginosa</i> (mm)		<i>Staphylococcus aureus</i> (mm)		<i>Bacillus subtilis</i> (mm)		<i>Proteus vulgaris</i> (mm)		<i>Salmonella typhimurium</i> (mm)		<i>Escherichia coli</i> (mm)		<i>Klebsicela pneumonia</i> (mm)	
	<i>in vitro</i> material	<i>in vivo</i> material	<i>in vitro</i> material	<i>in vivo</i> material	<i>in vitro</i> material	<i>in vivo</i> material	<i>in vitro</i> material	<i>in vivo</i> material	<i>in vitro</i> material	<i>in vivo</i> material	<i>in vitro</i> material	<i>in vivo</i> material	<i>in vitro</i> material	<i>in vivo</i> material
0.1 mg/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5 mg/ml	6.66 ± 0.57	0	0	6.66 ± 0.57	0	0	6.66 ± 0.57	0	0	0	0	6.66 ± 0.57	6.66 ± 0.57	0
1.0 mg/ml	7 ± 1	6.66 ± 0.57	0	7 ± 1	0	0	7 ± 1	0	0	0	6.66 ± 0.57	7 ± 1	7 ± 1	6.66 ± 0.57
5.0 mg/ml	8.33 ± 1.55	7 ± 1	0	8 ± 1	0	7 ± 1	8 ± 1	0	0	7 ± 1	7.33 ± 0.57	8.33 ± 1.52	8.33 ± 1.15	7 ± 1
10 mg/ml	10 ± 1.73	8 ± 1	0	8 ± 1	0	10 ± 1	8.66 ± 1.15	0	0	9 ± 1	10 ± 1	9.66 ± 0.57	10 ± 1	8 ± 1
20 mg/ml	11.66 ± 1.15	9 ± 1.73	0	9 ± 1.73	0	10.33 ± 0.57	10 ± 1	6.33 ± 0.57	0	9.66 ± 0.57	10.66 ± 1.15	11.66 ± 0.57	11.33 ± 0.57	10.33 ± 1.15
40 mg/ml	11.66 ± 1.52	10.33 ± 0.57	7 ± 1	11.66 ± 2.08	6.33 ± 0.57	12.33 ± 1.15	11.66 ± 1.15	7 ± 1	6.66 ± 0.57	10.66 ± 0.57	11.66 ± 1.15	14 ± 1	12 ± 1	11.66 ± 1.15
60 mg/ml	13.66 ± 0.57	11.66 ± 1.52	8.33 ± 0.57	13.66 ± 1.15	8.66 ± 1.15	13.33 ± 1.15	12.66 ± 1.15	8.33 ± 0.57	7 ± 1	12.66 ± 0.57	13.66 ± 1.52	15.66 ± 1.15	13.33 ± 0.57	13.66 ± 1.52
80 mg/ml	15.33 ± 0.57	13.33 ± 1.15	9.66 ± 0.57	15.66 ± 0.57	9.66 ± 1.15	16 ± 1	14.33 ± 1.52	10 ± 1	8.66 ± 1.15	14.33 ± 1.52	15.33 ± 0.57	17 ± 1	14 ± 1	16.66 ± 1.52
100mg/ml	17 ± 1	14.33 ± 1.52	11.33 ± 0.57	17.33 ± 1.52	12 ± 1	17.66 ± 1.52	15.66 ± 0.57	11.66 ± 1.52	10 ± 1	15.66 ± 0.57	16.33 ± 0.57	19.33 ± 3.05	15.33 ± 1.52	20.66 ± 2.08

(Note: all the compounds show positive result for positive and negative result for negative control; the diameter of paper disc is 6mm).

4. Conclusion

From the present investigation it can be concluded that callus of *W. somnifera* contained the antimicrobial compounds. Callus was effective in three strains of bacteria whereas natural root was effective in four strains of bacteria. Out of tested seven strains of bacteria, *in vitro* grown callus and *in vivo* materials contain similar chemical groups and inhibited the growth of bacteria in different concentrations. It can be concluded that, callus and natural root contain similar phytochemical compounds but in different concentrations.

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