Effect of commercial biofertilizers on growth and yield of antimalarial compounds of *Artemisia annua*

ABSTRACT

Introduction: *Artemisia annua* is a medicinal plant grown by Ugandan farmers in Kabale and Kabarole. It is particularly used in preparation of antimalarial herbal teas used to control and prevent malaria. The content of these antimalarial compounds in *A.annua* (Anamed) grown in Uganda are low compared to cultivars in other countries but could be enhanced using economical, eco-friendly, and sustainable fertilizers (biofertilizers). Thus, the effect of commercially available biofertilizers in Uganda on *A.annua* antimalarial components was assessed.

Methodology: Seven different commercially available biofertilizers containing nitrogen fixing strains and phosphate solubilizers obtained from the top biofertilizer distributors to Uganda (Shittah Agri and Biotech Pvt Ltd4 106 C22) and manufacturer (Kinyara Sugar Works Ltd.) were used. Their efficiency was assessed by measuring the vegetative growth characters and the content of flavonoids and artemisinin (using High Performance Liquid Chromatography, HPLC) and polysaccharides (using Ultra Violet visible, UV-vis spectrophotometer).

Results: Though overall increase in growth was observed upon application of the biofertilizers, there was variation in the concentration of the antimalarial compounds. The highest kaempferol content and artemisinin content were observed in *A. annua* inoculated with *Azotobacter spp*. AZT (17.05 µg/ml and 1.43%) and *Bacillus subtilis*, BS (19.12 µg/ml, 1.42%) while the highest quercetin content was observed in *A. annua* inoculated with AZT (0.42 µg/ml) and *Bacillus megaterium*, BM (0.41 µg/ml). Additionally, fertilizers (biofertilizers and chemical fertilizers) decreased polysaccharide content.

Conclusion: The findings demonstrate that application of commercially available biofertilizers in Uganda is beneficial in increasing concentration of artemisinin and flavonoids and thus can be utilized by *Artemisia annua* farmers in Uganda.

Key words: Artemisia annua, biofertilizers, flavonoids, polysaccharides, commercial, artemisinin

1. INTRODUCTION

Malaria is an infectious disease caused by different species of Plasmodium parasite and remains one of the leading causes of morbidity and mortality in Uganda [39]. In Uganda, artemisinin-based combination therapies (ACTs) are the most important malaria drugs used to treat patients suffering from malaria. However, the use of ACTs has been limited by many factors such as partial resistance of *Plasmodium falciparum* to artemisinin in the ACTs [10]thus leading to delayed clearance after therapy. Furthermore, poverty as many patients cannot afford them or do not have transport to take them to facilities with free services. Thus many resort to use available herbal remedies to treat or control malaria. Herbal teas made from *A. annua* such as artavol contain a mixture of antimalarial compounds and are less likely to face resistance.

In Uganda, *A. annua* was introduced around 2003 [14] and is mainly grown in Wakiso, Kaberamaido, Kapchorwa, Rukungiri, Kabarole and Kabale districts. The plant contains many antimalarial compounds such as artemisinin, flavonoids and polysaccharides [11, 26]. However, the content of these compounds is low compared to other countries for example artemisinin concentration is below 1% yet in other parts of the world it is up to 2% [19]. Thus, considering the importance of this plant, considerable investigations have been carried out to find ways of increasing artemisinin production in the plant [25]as chemical synthesis of artemisinin is expensive and quite complex. Furthermore, little work has been done to enhance the other antimalarial compounds present in the plant [25]yet compounds like essential oils could also be used as mosquito repellants. Therefore, this study was carried out to investigate the effect of commercially available biofertilizers (in the Ugandan market) on the growth and concentration of antimalarial compounds in *A. annua*. These form of fertilizers could easily be accepted by*A. annua* farmers as they have also been used to enhance growth in other crops in Uganda and thus would not face the problem of ecological adaptability which is one of the factors affecting biofertilizer usage.

Biofertilizers refers to formulations based on beneficial microbes that either fix atmospheric nitrogen or enhances the solubility of soil nutrients such as phosphorus and have potential to increase the yield of crops [23].They promote plant growth through various mechanisms such as nitrogen fixation, nutrient solubilization and mobilization, phytohormone production, microbial community diversification, and soil physicochemical property improvement [32].In comparison to chemical fertilizers that are costlyand are not environmentally friendly, biofertilizers are economical, eco-friendly and sustainable[32].In relation to *A. annua* plant, the microbes that have been utilized to enhance its active compounds are mainly fungi and a few bacteria [15].Among the common bacteria used as biofertilizers in *A.annua* are Azotobacter, Azospirillum, Bacillus, Pseudomonas, Streptomyces spp., Radiobacter spp., Stenotrophomonas spp [15]. In Uganda, there is lack of awareness of biofertilizersamongst farmers [32] hence in this study, the commercially available biofertilizers constituting of bacterial species mainly were used as they could easily be accepted by farmers as they have used them in enhancing growth in other plants.

2. METHODOLOGY

Description of the Experimental Area

The pot and field experiment was conducted at Tooro Botanical Garden (TBG), Kabarole district. Geographically, the experiment was conducted at a place located at N0040.018, E 03017.096 at an altitude of 1525m. The site receives mean annual rain fall of 1400mm per annum and the annual temperature is 220C. The physical properties of the soil used in the field and pot experiment were pH (5.9, 6.2), N (643.81, 621.28 kg/ha), P (28.4, 26.7 Kg/ha), K (304.82, 291.54 Kg/ha), OM (6.12, 5.05 %) and texture (Sandy loam and Sandy loam). Composition of bacterial community was analyzed by amplicon sequencing of 16S rRNA genes on an Illumina Miseq platform and the phylum Proteobacteria (34.2%) was the most prevalent followed by Acidobacteria (17.3%) and Actinobacteria (15.5%) [1] thus proteobacteria were used in the study.

Treatments

The study design was a Randomized Complete Block Design. Commercially available species were used in the study. These species have shown adaptability to the area as they have been used by farmers to enhance growth in other food crops and thus could easily be accepted by farmers as there is inadequate biofertilizer awareness amongst the smallholder farmers in Uganda [32]. The treatments included; NF-no fertilizer (negative control) and positive controls (U-Urea and TSP- Triple Super Phosphate) Species used were from Kinyara Sugar Works Ltd (PF-K- *Pseudomonas fluorescences*) and from the top most importer of biofertilizers to Uganda (Shittah Agri and Biotech Pvt Ltd4 106 C22) and they include AZT-Azotobacter spp., AZS-Azospirillum spp., BS- *Bacillus subtilis*, PF-S- *Pseudomonas fluorescences*, BM-*Bacillus megaterium* and BM+G- *Bacillus megaterium* and Glomus spp.

Propagation, Transplantation, Growth and Harvesting of *A. annua*

For, each treatment, 80 plants were planted (40 in the field and 40 in the pots). The microbial inoculants were applied to the soil before transplanting at a rate of 2 ml (containing 2 x 108 CFU/Ml) per hole [2]. The urea and TSP were applied in the soil before transplanting at a rate of 80 kg ha-1 and 40 kg ha-1 [20]. Stem cuttings of *A.annua* L. (Anamed) were used to prepare seedlings at TBG nursery. One-month old seedlings were then transplanted to pots and field. The row to row distance was 2m and plant to plant distance was 1m. At harvesting (four months after transplanting), the vegetative growth characters (VGCs) were determined i.e. the number of branches were counted and the plant height was measured. The average dry weight of dried leaves was determined.

Preparation of Extracts and Phytochemical Screening for Secondary Metabolites

Different solvents (water, diethyl ether and ethanol) were used for extraction. A decoction was prepared by boiling 10g of the dried leaves with 200 ml of distilled water for 15 minutes. The cooled samples were filtered through mucilin cloth and then again through cotton and were concentrated at 50 0C and lyophilized to dryness. An ethanol and diethyl ether extract were prepared by adding 10 grams of the dried leaves to 100 ml of 90% ethanol and diethyl ether. The samples were agitated on a shaker at a speed of 120 rpm for 1 hour (ethanol extract) and for 15 minutes (diethyl ether extract). The sample was then incubated for 24 hours. The samples were filtered and concentrated in vacuo to dryness using a rotary evaporator at 40 0C (ethanol extract) and 25 0C (diethyl ether extract).

The aqueous, ethanol and diethyl ether extracts were screened for the various phytochemicals following the procedures described by [5]. Using standards (artemisinin and kaempferol), thin layer chromatography (TLC) of the diethyl ether and ethanol extracts was conducted using the method described by [18]. Using artemisinin, kaempferol and quercetin standards, reverse-phase HPLC assay was conducted to estimate the artemisinin and flavonoid content in the diethyl ether and ethanol extracts. The content of polysaccharides in the aqueous extract was estimated using UV/VIS spectrophotometer following the phenol-sulphuric acid assay [29].

3. RESULTS AND DISCUSSION

Phytochemical screening of *Artemisia annua* extracts

The qualitative analysis for phytochemicals showed that glycosides, cardiac glycosides, phenols, phytosterols, steroids and coumarins were present in all extracts while anthraquinones, saponins and chalcones were absent in all extracts (Table 1). The tested phytochemicals were more in the ethanol extract followed by aqueous extract and lastly diethyl ether extract.

Table 1: Results of Qualitative Phytochemical Chemical Analysis

	GL	CG	PH	PS	STE	COU	ALK	POP	ANT	TAN	FLA	AQU	SAP	CHA	XAN	TER	TRI
DE	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+
EE	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+
WE	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-

SOLVENTS: DE-Diethyl ether extract EE-Ethanol extract WE-Water extract, PHYTOCHEMICALS- GL- Glycosides, CG-Cardiac glycosides, PH- Phenols, PS- Phytosterols, STE Steroids, COU- Coumarin, ALK- Alkaloids, POP-Polyphenols, ANT- Anthocyanins, TAN- Tannins, FLA-Flavonoids, AQU- Anthraquinones, SAP- Saponins,

CHA-Chalcones, XAN- Xanthoproteins, TER- Terpenoids, TRI- Triterpenoids+ Present and - Absent

TLC Analysis

Figure 1 and figure 2 represent the TLC results for the diethyl ether extract and ethanol extract respectively. For each TLC, 16 spots were observed. For the ethanol extracts, kaempferol and artemisinin were observed at retention factor (Rf) of 0.79 and 0.91 respectively. For the diethyl ether extract, no flavonoids were observed but artemisinin was observed at Rf of 0.75.

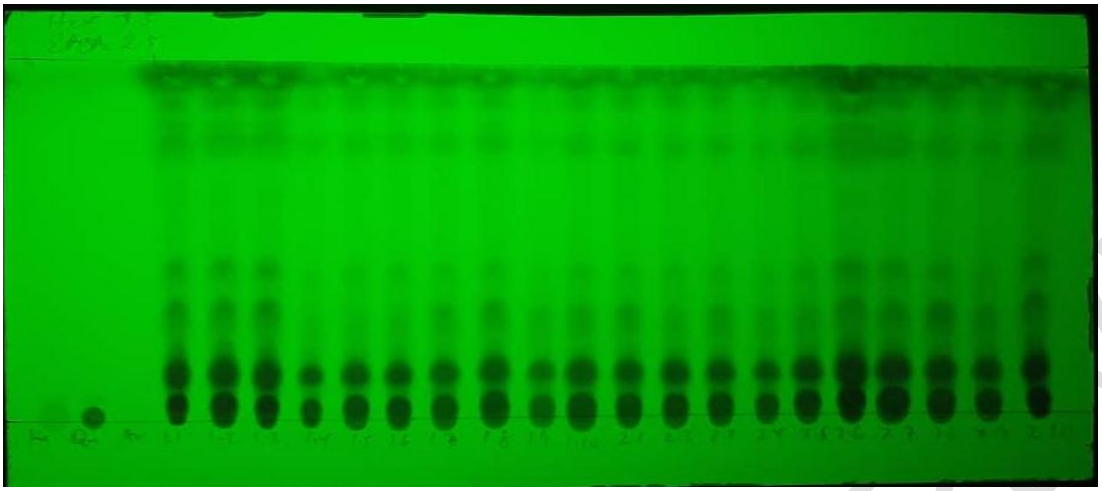

Figure 1: **TLC picture of the diethyl ether extract obtained using a mobile phase consisting of hexane and ethyl acetate (7.5: 2.5).** Ka-Kaempferol, Ar-Artemisinin, 1.X-Pot experiment, 2.X-Field experiment, X=1-10 where 1-no fertilizer (negative control), 2-Azotobacter, 3-Azospirillum, 4-Urea, 5- Triple Super Phosphate, 6- *Bacillus subtilis*, 7-*S: Pseudomonas fluorescences (*Shittah*),* 8: *Pseudomonas fluorescences (*Kinyara*)*, 9- *Bacillus megaterium* and 10- *Bacillus megaterium* and *Glomus* spp.

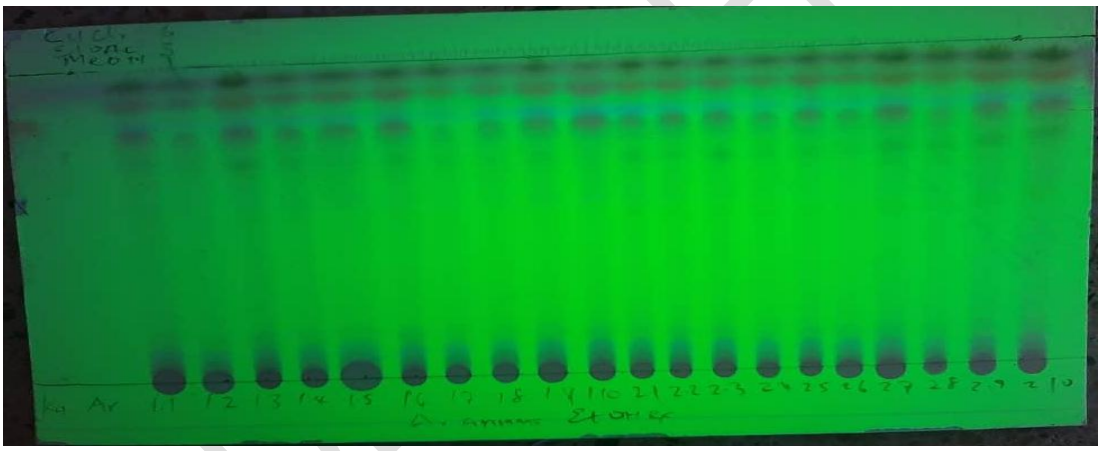

Figure 2: TLC Picture of the ethanol extract obtained using a mobile phase consisting of chloroform, ethyl acetate and methanol (6:3:1). Ka-Kaempferol, Ar-Artemisinin,1.X-Pot experiment, 2.X-Field experiment, X=1-10 where 1-no fertilizer (negative control), 2-Azotobacter, 3-Azospirillum, 4-Urea, 5- Triple Super Phosphate, 6- *Bacillus subtilis*, 7-*S: Pseudomonas fluorescences (*Shittah*),* 8: *Pseudomonas fluorescences (*Kinyara*)*, 9- *Bacillus megaterium* and 10- *Bacillus megaterium* and *Glomus* spp.

Effect of Microbes on Vegetative Growth Characters (VGC) of *A. annua*

The effect of nitrogen fixing bacteria (NFB) and phosphate solubilizing bacteria (PSB) on VGC of *A. annua* is illustrated in Table 2 and 3 respectively.

Effect on plant height: There were significant differences in plant height between the species grown with NFB and the negative control but not positive control (urea). Generally, plants inoculated with AZS showed the highest plant height in both the field (1.86 m) and pot experiment (1.74m). The species grown with PBS showed no significant differences in the pot experiment. On the other hand, in the field experiment, there was slight significant differences in plant height and most

species showed higher height than the negative control and *A. annua* inoculated with BM showed the highest plant height (1.9 m).

Effect on dry weight: Results showed that in both the pot and field experiment, the dry weight of plants inoculated with NFB or PSB was higher than the plants grown without fertilizers. However, the positive control plants (urea) had higher dry weight than the plants inoculated with NFB. The plants inoculated with AZT (21.9, 92.7 g) had higher dry weight than plants inoculated with AZS (20.6, 90.7 g). On the other hand, plants inoculated with PF species had a higher dry weight than the positive controls and were followed by plants inoculated with BS species.

Effect on the number of branches: Both the pot and field experiment showed that the number of branches of plants inoculated with NFB or PSB was higher than the plants grown without fertilizers. However, the positive control plants (TSP) on average had higher branches than plants inoculated with NFB. The plants inoculated with AZS (16.33, 98.33) had higher branch numbers than plants inoculated with AZT (12.67, 76). On the other hand, TSP (42, 126.67) also had higher branch numbers than the plants inoculated with PSB except those inoculated with a co-inoculation (BM+G, 44.33, 191).

Table 2: Effect of Nitrogen Fixing Bacteria on Vegetative Growth Characters of*A.annua*

	NF	AZT	AZS	U
Pot Experiment				
Dry weight (g,/plant)*	15	21.9	20.6	25.4
Plant height (m)	1.65 ± 0.0^{b}	1.7 ± 0.0^{ab}	1.74 ± 0.1^{a}	1.74 ± 0.0^{a}
Number of Branches	11.67 ± 0.6^{b}	12.67 ± 0.6^{b}	16.33 ± 2.3^{b}	29 ± 6.1^{a}
Field Experiment				
Dry weight (g,/plant)*	88.2^{c}	92.7^{b}	90.7^{b}	107.85^{a}
Plant height (m)	1.73 ± 0.0^{b}	1.72 ± 0.0^{b}	1.86 ± 0.1^{a}	1.77 ± 0.0^{ab}
Number of Branches	54.33 ± 7.5^{c}	76 ± 8.0^{bc}	98.33 ± 3.5^{ab}	126 ± 28.9^{a}

Mean ± standard deviation; n = 3; *-Total leaf dry weight per plant, Values within a row with same superscripted letter are not significantly different at P>0.05; NF-no fertilizer (negative control), AZT-Azotobacter, AZS-Azospirillum, U-Urea

Table 3: Effect of Phosphate Solubilizing Bacteria on Vegetative Growth Characters of *A. annua*

	NF	BS	PF-S	PF-K	BM	BM+G	TSP
Pot Experiment							
Dry weight (g,/plant)*	15	18.6	19.5	19	18.1	18.2	17.4
Plant height (m)	1.75 ± 0.0^{a}	1.76 ± 0.0^{a}	1.76 ± 0.0^{a}	1.75 ± 0.0^{a}	1.75 ± 0.0^{a}	1.74 ± 0.0^{a}	1.77 ± 0.0^{a}
Number of Branches	11.67 ± 0.6^{e}	20.67 ± 1.2^{c}	13.67 ± 1.5^{e}	14 ± 1.0^{e}	29 ± 1.7^{d}	44.33 ± 3.2^{a}	42 ± 1.7^{a}
Field Experiment							
Dry weight	88.2	89.5	92.5	91.5	88.5	88	91

(g,/plant)*							
Plant height (m)	1.73 ± 0.0^{bc}	1.7 ± 0.0^{bc}	1.66 ± 0.2^{c}	1.78 ± 0.1^{b}	1.9 ± 0.1^{a}	1.84 ± 0.0^{b}	1.85 ± 0.1^{b}
Number of Branches	54.33 ± 7.5^{d}	94.33 ± 2.1^{c}	100.33 ± 10.2^{c}	105 ± 3.5 ^{bc}	110.67 ±11.1^{bc}	191 ± 7.8^{b}	126.67 ± 32.5^{a}

Mean ± standard deviation; n = 3; *-Total leaf dry weight per plant, Values within a row denoted by same letter are not significantly different (P>0.05). NF-No fertilizer, TSP- Triple Super Phosphate, BS- *Bacillus subtilis*, PF-S: *Pseudomonas fluorescences* (Shittah), PF-K: *Pseudomonas fluorescences* (Kinyara), BM- *Bacillus megaterium* and BM+G- *Bacillus megaterium* and *Glomus* spp.

Effect of Microbes on the concentration of Antimalarial Compounds of *A.annua*

In both field and pot experiment, there were significant mean differences between the artemisinin content in the *A. annua* grown with bio-fertilizers and the negative control (table 4 and table 5). Diethyl ether extracts were found to have a higher artemisinin than ethanol extracts. Furthermore, the results indicated that the concentrations of artemisinin present in *A. annua* grown in the pots were generally higher than the one observed in *A. annua* grown in the field. The highest artemisinin content in the diethyl ether and ethanol extracts was observed in *A. annua* inoculated with AZT (1.43, 0.40%) and BS (1.42, 0.37%) among the NFB and PSB treatments respectively.

Two standards (Quercetin and Kaempferol) were used to analyze flavonoids in the ethanol extracts of *A. annua* (Table 6 and 7). In both field and pot experiment, there were significant differences between the flavonoid content in the *A. annua* grown with biofertilizers and the negative and positive control. Kaempferol was found to have a higher concentration compared to quercetin. In addition, the concentrations of flavonoids present in *A. annua* grown in the pots were generally higher than those observed in *A. annua* grown in the field. The highest kaempferol content was observed in *A. annua* inoculated with AZT (17.05 µg/ml) and BS (19.12 µg/ml) among the NFB and PSB treatments respectively. The highest quercetin content was observed in *A. annua* inoculated with AZT (0.42 µg/ml) and BM (0.41 µg/ml) among the NFB and PSB treatments respectively.

Generally, in both the pot and field experiments, both NFB and PSB negatively affected polysaccharide content (Figure 3 and 4). The maximum polysaccharide content was with the negative control (62.02, 75.82 µg/ml). The lowest polysaccharide content was in plants inoculated with TSP (51.14 µg/ml). Nonetheless, the chemical fertilizers decreased the polysaccharide content more than NFB and PSB. The co-inoculation (BM+G) and AZT did not decrease the polysaccharide content significantly as their content had no significant differences with negative control in the field and pot experiment respectively.

Table 4: Effect of Nitrogen Fixing Bacteria on the Artemisinin content of *A.annua*

	NF	AZT	AZS	U
Pot Experiment (%)				
Diethylether*	1.19 ± 0.01^{d}	1.43 ± 0.00^{a}	1.25 ± 0.01^{c}	1.35 ± 0.00^{b}
Ethanol*	0.37 ± 0.00^{a}	0.39 ± 0.01^{a}	0.37 ± 0.04^{a}	0.27 ± 0.01^{b}
Field Experiment (%)				
Diethylether*	1.27 ± 0.00^{a}	1.23 ± 0.00^{b}	1.12 ± 0.00^{c}	1.12 ± 0.01^{c}
Ethanol*	0.31 ± 0.02^{b}	0.40 ± 0.00^{a}	0.26 ± 0.00^{c}	0.32 ± 0.00^{b}

*-Solvents Used in Extraction, Mean ± standard deviation; n = 2;Values within a row denoted by the same letter are not significantly different (P>0.05). NF-no fertilizer (negative control), AZT-Azotobacter, AZS-Azospirillum, U-Urea

Table 5: Effect of Phosphate Solubilizing Bacteria on the Artemisinin content of *A. annua*

	NF	BS	PF-S	PF-K	BM	BM+G	TSP
Pot Experiment (%)							
Diethylether*	1.19 ± 0.01^{e}	1.42 ± 0.00^{a}	1.39 ± 0.00^{ab}	1.24 ± 0.00^{d}	1.32 ± 0.00^{c}	1.35 ± 0.04^{bc}	1.37 ± 0.00^{b}
Ethanol*	0.37 ± 0.00^{ab}	0.41 ± 0.01^{ab}	0.32 ± 0.01^{b}	0.36 ± 0.01^{ab}	0.41 ± 0.01^{ab}	0.38 ± 0.03^{ab}	0.43 ± 0.10^{a}
Field Experiment (%)							
Diethylether*	1.27 ± 0.00^{a}	1.26 ± 0.00^{ab}	1.24 ± 0.02^{b}	1.1 ± 0.01^{d}	1.04 ± 0.00^{e}	1.28 ± 0.01^{a}	1.2 ± 0.02^{c}
Ethanol*	0.31 ± 0.02^{bc}	0.37 ± 0.01^{a}	0.31 ± 0.02^{bc}	0.31 ± 0.01^{bc}	0.30 ± 0.01^{c}	0.34 ± 0.01^{b}	0.34 ± 0.01^{b}

*-Solvents Used in Extraction, Mean ± standard deviation; n = 2;Values within a row denoted by the same letter are not significantly different (P>0.05). NF-no fertilizer (negative control), TSP- Triple Super Phosphate, BS- *Bacillus subtilis*, PF-S: *Pseudomonas fluorescences* (Shittah), PF-K: *Pseudomonas fluorescences* (Kinyara), BM- *Bacillus megaterium* and BM+G- *Bacillus megaterium* and *Glomus* spp

Table 6: Effect of Nitrogen Fixing Bacteria on the flavonoid content of *A.annua*

	NF	AZT	AZS	U
Pot Experiment (µg/ml)				
Kamferol*	16.47 ± 0.0^{b}	17.05 ± 0.1^{a}	16.04 ± 0.0^{c}	11.72 ± 0.0^{d}
Quercetin*	0.39 ± 0.0^{a}	0.38 ± 0.0^{b}	0.38 ± 0.0^{b}	0.38 ± 0.0^{b}
Field Experiment (µg/ml)				
Kamferol*	11.49 ± 0.0^{d}	14.51 ± 0.0^{a}	12.41 ± 0.1^{c}	13.74 ± 0.1^{b}
Quercetin*	0.39 ± 0.0^{b}	0.42 ± 0.0^{a}	0.34 ± 0.0^{c}	0.37 ± 0.0^{b}

*-Standard Used in Analysis, Mean ± standard deviation; n = 3; Values within a row denoted by the same letter are not significantly different (P>0.05). NF-no fertilizer (negative control), AZT-Azotobacter, AZS-Azospirillum, U-Urea

Table 7: Effect of Phosphate Solubilizing Bacteria on the flavonoid content of *A.annua*

	NF	BS	PF-S	PF-K	BM	BM+G	TSP
Pot Experiment (µg/ml)							
Kamferol*	16.47 ± 0.0^{d}	19.12 ± 0.0^{a}	11.10 ± 0.0^{g}	14.16 ± 0.1^{f}	14.29 ± 0.1^{e}	16.59± 0.0^{c}	17.0 ± 0.1^{b}
Quercetin*	0.39 ± 0.0^{c}	0.39 ± 0.0^{c}	0.36 ± 0.0^{e}	0.40 ± 0.0^{b}	0.41 ± 0.0^{a}	0.39 ± 0.0^{c}	0.37 ± 0.0^{d}
Field Experiment (µg/ml)							

Kamferol*	11.49 ± 0.0^{e}	14.21 ± 0.1^{d}	15.60 ± 0.1^{a}	11.03 ± 0.1^{g}	13.18 ± 0.1^{f}	14.37 ± 0.1^{c}	15.14 ± 0.0^{b}
Quercetin*	0.39 ± 0.0^{a}	0.33 ± 0.0^{c}	0.39 ± 0.0^{a}	0.35 ± 0.0^{b}	0.35 ± 0.0^{b}	0.33 ± 0.0^{c}	0.35 ± 0.0^{b}

*-Standard Used in Analysis, Mean ± standard deviation; n = 3; Values within a row denoted by the same letter are not significantly different (P>0.05).. NF-no fertilizer (negative control), TSP- Triple Super Phosphate, BS- *Bacillus subtilis*, PF-S: *Pseudomonas fluorescences* (Shittah), PF-K: *Pseudomonas fluorescences* (Kinyara), BM- *Bacillus megaterium* and BM+G- *Bacillus megaterium* and *Glomus* spp

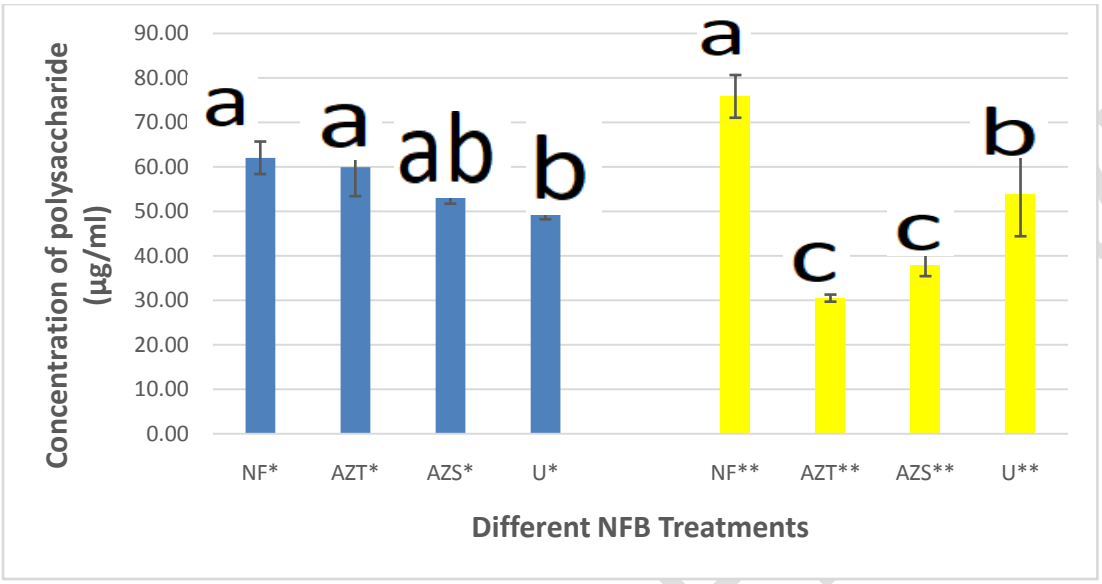

Figure 3: Effect of nitrogen fixing bacteria (NFB) on the polysaccharide content

Mean ± standard deviation; n = 3;*-pot experiment, **- Field experiment, Bars with the same * or color denoted by the same letter are not significantly different (P>0.05).NF-no fertilizer (negative control), AZT-Azotobacter, AZS-Azospirillum, U-Urea

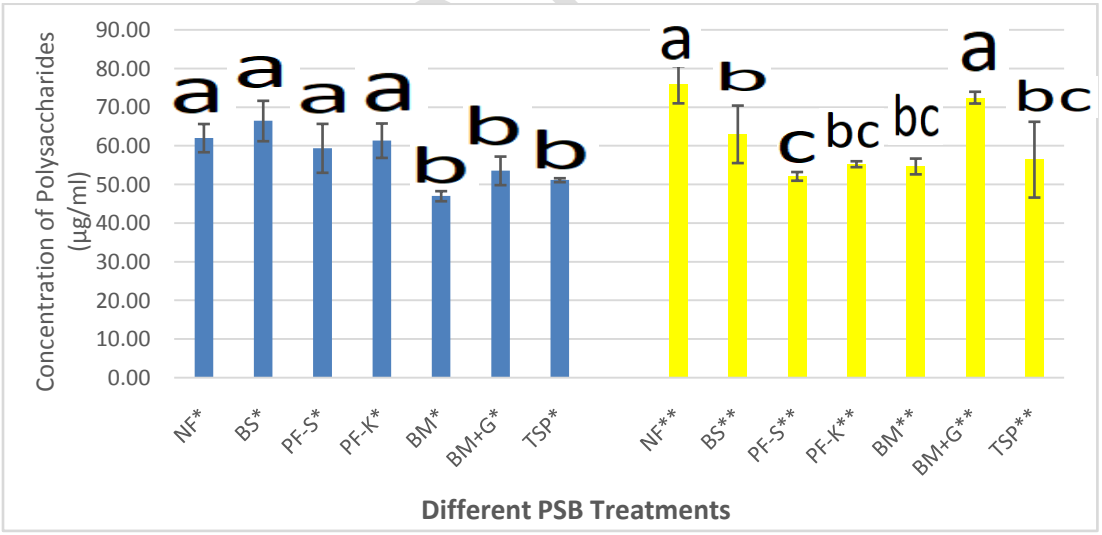

Figure 4: Effect of Phosphate solubilizing bacteria (PSB) on the polysaccharide content Mean ± standard deviation, (n=3). *-pot experiment, **- Field experiment, Bars with the same * or ** denoted by the same letter are not significantly different (P>0.05).NF-no fertilizer (negative control), TSP- Triple Super Phosphate, BS- *Bacillus subtilis*, PF-S: *Pseudomonas fluorescences* (Shittah), PF-K: *Pseudomonas fluorescences* (Kinyara), BM- *Bacillus megaterium* and BM+G- *Bacillus megaterium* and *Glomus* spp.

DISCUSSION

Fertilizers influenced vegetative growth characters (VGC) of *A. annua* but there were inconsistencies in their effects. In this study, both chemical and microbial inoculants increased *A. annua* VGC. The observation was in line with what has been reported by some authors who used bacterial inoculants [2,3, 4]and chemical fertilizers [12, 15, 27]. However, some authors observed no statistical effect on the *A.annua* plant VGC with varying chemical fertilizer doses [24,28].

The variation of phytochemicals observed could be attributed to the solvents and method used in extraction [44]. No flavonoids were detected in the TLC plate of the diethyl ether extract because of the less polarity of diethyl ether. Considering HPLC and TLC results, the concentration of artemisinin was higher in the diethylether extracts than ethanol extracts which was because of less polarity of artemisinin that had more affinity for less polar diethyl ether. Furthermore, [42] observed that solubility of artemisinin in water + ethanol mixtures increases with an increase in ethanol concentration indicating that extraction capacity increased with decreasing polarity.

Generally the microbial inoculants (NFB and PSB) increased the flavonoid and artemisinin content compared to the control. The increase in flavonoid content is in agreement with those obtained in *A. annua* inoculated with fungal species either singly [16]or in combination with bacteria [2]. Furthermore, the detection of kaempferol in leaves was similar to the observation of [6] but differed from those of [38] who never detected kaempferol in *A. annua*leave except in flowers. This may be attributed to the difference in method of extraction, sample handling, storage since flavonoids are liable to degradation, variety of *A. annua* and sample (dry leaves) used in this study. But on the other hand, though the parts were different, [38] observed more kaempferol than quercetin in *A. annua* flowers like we observed more kaempferol than quercetin in the *A.annua* leaves. The artemisinin range (0.26-1.43) observed in this study was in agreement with what has been reported by other authors though majority used mycorrhizal species either singly[7,21,34] or in combination with bacteria [4]. In this study, generally *Bacillus subtilis* and Azotobacter spp were observed to be the best PSB and NFB respectively. This may also confirm why they have been the most studied bacteria in relation to *A. annua*[30].

Increase in active compounds as a result of use of microbial inoculants could be attributed to a number of mechanisms such as altering microbial communities in soil [45], increasing nutrient use efficiency of nitrogen, phosphorus, and potassium [31] and increasing the soil organic matter, total nitrogen and phosphorus and available phosphorus and potassium contents [41]. Thus, the main way is nitrogen fixing in relation to the NFB but specific NFB have other means. Azotobacter species (AZT) release exopolysaccharides which improve the plant's nutritional benefits, bring about stress tolerance and fitness factor (resistance to pathogens) [17]. Azospirillum species (AZS) produce plant growth substances (such as indole acetic acid) that stimulate root formation hence better nutrient uptake [36, 40], this observation has also been reported in *A. annua* inoculated with a co-inoculation of AZT and AZS [33]. On the other hand, PSB increase available soil phosphate by producing organic acids or enzymes (phosphatases and phytases) which dissolve inorganic phosphates or dissociate phosphorus from organic sources respectively [40]. The good activity exhibited by *Bacillus subtilis*and *Azotobacter* spp. was expected as bacteria belonging to the genus *Bacillus* have been reported as the most common *A. annua* endophytic bacteria (live in host plant tissues and benefit the host plant without a harmful effect) and are known to improve host plant growth and enhance their resistance against abiotic and biotic stresses [46]while on the other hand, *Azotobacter* sppare among the species that have been successfully formulated into commercial biofertilizers as they are efficient nitrogen fixing strains that are non-symbiotic [47].

Furthermore, the fertilizers (chemical and biofertilizers) decreased the polysaccharide content of *A.annua*. This observation has been observed in other plants such as *Lycium barbarum*[8, 35]and *Triticum aestivum*[9, 22, 37]. In case of nitrogen fertilizers, the negative effect is because they affect polysaccharide synthesis [8]as their presence increases

the survival of the photosynthetically-active leaf for a longer period than usual [9]thus in the process, some the polysaccharides may be utilized. In relation to phosphorus fertilizer, [43] reported that P fertilizer application in wheat growing significantly affected starch accumulation by influencing the expression of genes related to starch biosynthesis and degradation. Thus, it may be speculated that incase of *A. annua*, P fertilizers may influence genes related to polysaccharide degradation which in the end results in low polysaccharide content.

Largely there was agreement and a few variations between the trends observed in the pot and field experiments. The pot values were lower than the field values for the VGC but the vice versa was true for the active compound content. This observation has also been reported by [15] and he attributed the low active compound (artemisinin) in the field plants to the excess fertilization (Nitrogen) at field levels compared to the specific fertilizer content in the pot. Excess/ less fertilization can occur during rainy season when there is leaching. Furthermore, pot experiments having lower VGC compared to the field experiments could be a result of limited nutrient uptake by the plant. This is because in pot experiments, the roots are restricted in expanding [15]and due to considerable fluctuations in soil water content during a pot experiment [13].

CONCLUSION

This work highlighted the importance of selecting the right bacteria to use as a biofertilizer as of the seven species tested, *Bacillus subtilis* and Azotobacter spp were efficient PSB and NFB respectively for *Artemisia annua* and thus farmers can apply them to increase the active compounds in the plant. Furthermore also chemical fertilizers showed positive results and in some cases were better than the biofertilizers thus confirming the issue that it is hard to outcompete them but could reduce the amount used through coinoculation (for example the amount of urea used in this study could be halved and the other half supplemented by Azotobacter) thus investigations are necessary to assess the effect of different coinoculations (mix of chemical fertilizers together with plant-growth-promoting bacteria) on the concentration of various A. annua components. On the other hand, when using fertilizers, precaution should be taken if interested in *A. annua* polysaccharides (known also as anticancer agents) as fertilizers reduce them.

REFERENCES

1. Aidah, N., Engeu, O.P., Baptist, T.J., Muwanika, V.B., Johnson, M. and Joel, B., 2023. Diversity of bacterial community in the rhizosphere and bulk soil of *Artemisia annua* grown in highlands of uganda.Plos one, 18(3), p.e0269662.
2. Arora, M., Saxena, P., Choudhary, D. K., Abdin, M. Z., & Varma, A. (2016). Dual Symbiosis Between Piriformospora Indica And Azotobacter Chroococcum Enhances the Artemisinin Content In *Artemisia annua* L. World Journal Of Microbiology And Biotechnology, 32(19).
3. Arora, M., Saxena, P., Abdin, M. ., & Varma, A. (2020). Interaction Between Piriformospora Indica And Azotobacter Chroococcum Diminish the Effect of Salt Stress In *Artemisia annua* L .By Enhancing Enzymatic And Non-Enzymatic Antioxidants. Symbiosis.80, 61–73
4. Awasthi, A., Bharti, N., Nair, P., Singh, R., Ashutosh, K. S., Gupta, M. M., … Kalra, A. (2011). Synergistic Effect of Glomus Mosseae and Nitrogen Fixing Bacillus Subtilis Strain Daz26 on Artemisinin Content in *Artemisia annua* L. Applied Soil Ecology, 49, 125–130.
5. Balamurugan, V., Fatima, S., & Velurajan, S. (2019). A Guide to Phytochemical Analysis. International Journal of Advance Research and Innovative Ideas in Education, 5(1), 236-245.
6. Carvalho, I. S., Cavaco, T., & Brodelius, M. (2011). Phenolic Composition And Antioxidant Capacity Of Six Artemisia Species. Industrial Crops and Products, 33(2), 382–388.
7. Chaudhary, V., Kapoor, R., & Bhatnagar, A. (2008). Effectiveness Of Two Arbuscular Mycorrhizal Fungi On Concentrations Of Essential Oil And Artemisinin In Three Accessions Of *Artemisia Annua* L. Applied Soil Ecology, 40(1), 174–181.
8. Chung, R., Chen, C., & Ng, L. (2010). Nitrogen Fertilization Affects The Growth Performance, Betaine And Polysaccharide Concentrations of Lycium Barbarum. Industrial Crops &Products, 32(3), 650–655.
9. Coles, G. D., Jamieson, P. D., Hay, A. J., Atwell, W. A., & Fulcher, R. G. (1997). Environmentally-Induced Variation In Starch And Non-Starch Polysaccharide Content In Wheat. Journal Of Cereal Science 48, 26, 47–54.
10. Conrad, M.D., Asua, V., Garg, S., Giesbrecht, D., Niaré, K., Smith, S., Namuganga, J.F., Katairo, T., Legac, J., Crudale, R.M. And Tumwebaze, P.K., 2023. Evolution Of Partial Resistance To Artemisinins In Malaria Parasites In Uganda. New England Journal of Medicine, 389(8), Pp.722-732.
11. Daddy, N. B., Kalisya, L. M., Bagire, P. G., Watt, R. L., Towler, M. J., & Weathers, P. J. (2017). *Artemisia annua* Dried Leaf Tablets Treated Malaria Resistant to Act and Iv Artesunate. Phytomedicine, 32, 37-40.
12. Davies, M. J., Atkinson, C. J., Burns, C., Woolley, J. G., Hipps, N. A., Arroo, R. J., … Bentley, S. (2009). Enhancement of Artemisinin Concentration and Yield In Response to Optimization Of Nitrogen and Potassium Supply To *Artemisia annua*, Annals Of Botany, 104 (2), 315–323.
13. De Vries, M. P. C. (1980). Communications In Soil Science And Plant Analysis How Reliable Are Results Of Pot Experiments ? Communications In Soil Science And Plant Analysis, 3624(December), 895–902
14. Engeu, P. O., Omujal, F., Agwaya, M., Kyakulaga, H., & Obua Celestino. (2015). Variations in Antimalarial Components Of *Artemisia annua* Linn From Three Regions Of Uganda. African Health Sciences, 15(3), 828–834.
15. Ferreira, J. F. S. (2007). Nutrient Deficiency In The Production Of Artemisinin , Dihydroartemisinic Acid , And Artemisinic Acid In *Artemisia annua* L. Journal Of Agricultural And Food Chemistry, 55, 1686–1694.
16. Fortin, S.,& Melchert, V. (2015). Effect of Mycorrhizae On *Artemisia annua*. Project Report Submitted To Worcester Polytechnic Institute, 1–47.
17. Gauri, S. S., Mandal, S. M., & Pati, B. R. (2012). Impact of Azotobacter Exopolysaccharides On Sustainable Agriculture. Applied Microbiology And Biotevhnology, 95, 331–338

18. Ghafoori, H., Sariri, R., Naghavi, M. R., Aryakia, E., Dolatyari, A., Fazeli, S. S.,.. & Farahmand, Z. (2013). Analysis Of Artemisinin Isolated From *Artemisia Annua* L. By Tlc And Hplc. Journal Of Liquid Chromatography & Related Technologies, 36(9), 1198-1206.

19. Ikram, N. K. B. K., & Simonsen, H. T. (2017). A Review Of Biotechnological Artemisinin Production In Plants. Frontiers In Plant Science, 8, 1–10.

20. Jha, P., Ram, M., Khan, M. A., Kiran, U.,& Abdin, M. Z. (2011). Impact of Organic Manure and Chemical Fertilizers on Artemisinin Content and Yield in *Artemisia annua*L. Industrial Crops And Products, 33(2), 296-301.

21. Kapoor, R., Chaudhary, V., & Bhatnagar, A. K. (2007). Effects Of Arbuscular Mycorrhiza And Phosphorus Application On Artemisinin Concentration In *Artemisia annua* L . Mycorrhiza, 17, 581–587.

22. Kindred, D. R., Verhoeven, T. M. O., Weightman, R. M., Swanston, J. S., Agu, R. C., Brosnan, J. M., & Sylvester-Bradley, R. (2008). Effects Of Variety And Fertiliser Nitrogen On Alcohol Yield , Grain Yield , Starch And Protein Content , And Protein Composition Of Winter Wheat. Journal of Cereal Science 48, 48, 46–57.

23. Kumar, V. (2014). Characterization, Bio-Formulation Development and Shelf-Life Studies Of Locally Isolated Bio-Fertilizer Strains. Octa Journal Of Environmental Research, 2(1), 32–37

24. Lulie, B., Nigussie, A., & Chala, M. (2017). Response Of Artemisia (*Artemisia annua* L .) To Nitrogen And Phosphorus Fertilizers In Wondo Genet And Koka , Ethiopia. Journal of Agricultural Science And Research, 5(6), 407–413.

25. Namuli, A., Bazira, J., Casim, T.U. And Engeu, P.O., 2018. A Review Of Various Efforts To Increase Artemisinin And Other Antimalarial Compounds In Artemisia Annua L Plant. Cogent Biology, 4(1), P.1513312.

26. Ogwang, P. E., Ogwal, J. O., Kasasa, S., Olila, D., Ejobi, F., Kabasa, D., & Obua, C. (2012). *Artemisia annua* L . Infusion Consumed Once A Week Reduces Risk Of Multiple Episodes Of Malaria : A Randomised Trial In A Ugandan Community. Tropical Journal Of Pharmaceutical Research, 11(3), 445–453.

27. Omer, E., Hussein, A., El-Din, E., Azza, A., & El-Gendy, A. . (2014). Effect Of Nitrogen And Potassium Fertilizers On Growth , Yield , Essential Oil Effect Of Nitrogen And Potassium Fertilizers On Growth , Yield , Essential Oil And Artemisinin Of *Artemisia annua* L Plant. International Research Journal Of Horticulture, 2(2), 11–20.

28. Ozguven, M., Sener, B. ¸, Orhan, I., Sekeroglu, N., Kirpik, M., Kartal, M., … Kaya, Z. (2008). Effects Of Varying Nitrogen Doses On Yield , Yield Components And Artemisinin Content Of *Artemisia annua* L . Industrial Crops And Products, 27, 60–64.

29. Pawar, H. A., & Mello, P. M. (2011). Spectrophotometric Estimation Of Total Polysaccharides In Cassia Tora Gum. Journal Of Applied Pharmaceutical Science, (Issue), 93-95.

30. Peterfi, O., & Domokosi, E. (2018). Mutualistic And Endophytic Microorganisms Of *Artemisia annua* : Description , Role And Use. Acta Biologica Marisiensisis, 12(December), 5–21.

31. Rahman, W., Lelono, A. A., Hafiizh, E. A., & Ermayanti, T. M. (2022). Biofertilizer Increases Nutrient Use Efficiency (Nue) Of Nitrogen, Phosphorus, And Potassium At Leaves Level Of *Artemisia annua* L. In Aip Conference Proceedings (Vol. 2462, No. 1, P. 060002). Aip Publishing Llc.

32. Raimi A, Roopnarain A, Adeleke R. Biofertilizer Production In Africa: Current Status, Factors Impeding Adoption And Strategies For Success. Scientific African. 2021 Mar 1;11:E00694.

33. SHAKOURI, M. J., ABADI, A. F., NOORALVANDI, T., & KESHAVARZI, M. H. B. (2014). STUDY THE EFFECT OF BIOLOGICAL AND CHEMICAL FERTILIZERS ON ARTEMISIA ANNUA L . ROOT CHARACTERISTICS. INTERNATIONAL JOURNAL OF BIOSCIENCES, 4(12), 295–300.

34. Sharma, G., & Agrawal, V. (2013). Marked Enhancement In The Artemisinin Content And Biomass Productivity In Artemisia Annua L. Shoots Co-Cultivated With *Piriformospora Indica*. World Journal Of Microbiology And Biotechnology, 29(6), 1133–1138.

35. Shi, Z., Wei, F., Wan, R., Li, Y., Wang, Y., An, W., … Feng, J. (2019). Impact Of Nitrogen Fertilizer Levels On Metabolite Profiling Of The *Lycium Barbarum* L. Fruit. Molecules, 24, 1–14.

36. Sivasakthivelan, P. And Saranraj, P., 2013. Azospirillum And Its Formulations: A Review. International Journal Of Microbiological Research, 4(3), Pp.275-287.

37. Świtek, S., Takacs, V., Sawinska, Z., & Kosiada, T. (2019). Mineral Nitrogen Fertilisers Remain A Crucial Factor Even In The Ecological Intensification Of Agriculture. Acta Agriculturae Scandinavica,4710, 1–6.

38. Song, Y., Taye, K., Kim, G., Jung, S., Sup, W., Kim, Y., … El-Aty, A. M. A. (2016). Polyphenolic Profile And Antioxidant Effects Of Various Parts Of *Artemisia annua* L . Biomedical Chromatography, 30, 588–595.

39. Uganda Ministry Of Health, Annual Health Sector Performance Report FinancialYear 2020/21. 2021, Ministry Of Health: Kampala

40. Uribe, D., Sanchez-Nieves, J., & Vanegas, J. (2010). Role Of Microbial Biofertilizers In The Development Of A Sustainable Agriculture In The Tropics. In Soil Biology And Agriculture In The Tropics (Pp. 235–250).

41. Wang, J., Liu, L., Gao, X., Hao, J. And Wang, M., (2021). Elucidating The Effect Of Biofertilizers On Bacterial Diversity In Maize Rhizosphere Soil. Plos One, 16(4), P.E0249834.Wei, X., Bai, X., Cao, P., Wang, G., Han, J. And Zhang, Z., 2023. Bacillus And Microalgae Biofertilizers Improved Quality And Biomass Of Salvia Miltiorrhiza By Altering Microbial Communities. Chinese Herbal Medicines, 15(1), Pp.45-56.

42. Wang, L., Song, Y., Chen, Y., & Cheng, Y. (2007). Solubility of Artemisinin in Ethanol + Water from (278 . 2 to 343 . 2) K. *J.Chem. Eng. Data*, *52*, 757–758.
43. Zhang, R., Li, C., Fu, K., Li, C., & Li, C. (2018). Phosphorus Alters Starch Morphology And Gene Expression Related To Starch Biosynthesis And Degradation In Wheat Grain, Frontiers In Plant Science, 8, 2252.
44. Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. Clinical Microbiology Reviews, 12(4), 564–582.

45. Wei, X., Bai, X., Cao, P., Wang, G., Han, J. And Zhang, Z.,(2023). Bacillus and microalgae biofertilizers improved quality and biomass of salvia miltiorrhiza by altering microbial communities. Chinese herbal medicines, 15(1), PP.45-56.

46. Hadian, S. And Supronienė, S., (2023). The potential of Artemisia spp. Plant extracts and endophytic bacteria to increase plant productivity: a review. Zemdirbyste-agriculture, 110(1).

47. Aloo, B.N., Tripathi, V., Makumba, B.A. and Mbega, E.R., (2022). Plant Growth-Promoting Rhizobacterial Biofertilizers For Crop Production: The Past, Present, And Future. Frontiers In Plant Science, 13, P.1002448.