Evaluation of the Antibacterial Activity of Miswak (*Salvadora persica*) and Persian Lime (*Citrus latifolia*) Extracts Against *Escherichia coli* and *Staphylococcus aureus*

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Abstract: *Despite advances in medicine, the phenomenon of emerging drug resistance provokes novel research on active botanical compounds and alternative therapy development. Bioactive compounds present in plants possess potent antibacterial properties. The current study aims to evaluate and compare the antibacterial activity between miswak (Salvadora persica) and Persian lime (Citrus latifolia) extracts, a novel combination. Miswak sources unique phytochemicals, making it a superior tool for oral hygiene, while the volatile oil harbored within lime is traditionally used as a flavoring and cosmetic agent. Crude extraction of phytochemicals was done via cold maceration, employing polar solvents methanol and ethanol. Varying concentrations (150 mg/mL and 200 mg/mL) of extracts were subjected to antibiotic susceptibility testing (ABST) using agar well diffusion, while gentamicin and vancomycin served as the positive controls. Both Escherichia coli and Staphylococcus aureus exhibited susceptibility toward all extracts that were assayed. Triplicate readings were statistically analyzed using two-way analysis of variance (ANOVA) and student's t-test with 95% confidence interval ($p \leq 0.05$). Mean zones of inhibition (ZOI) varied, ranging from 10.7±0.6 mm to 13.7±0.6 mm for miswak and 16.7±0.6 mm to 19.7±1.2 mm for lime. Methanolic lime of 200 mg/mL (M/L2) demonstrated a pronounced ZOI against E. coli (19.7±1.2 mm), proving its supremacy over miswak. Upon further testing, lime extracts displayed a minimum inhibitory concentration (MIC) at 12.5 mg/mL and a minimum bactericidal concentration (MBC) at 25 mg/mL. Nonetheless, based on overall results, both miswak and lime extracts serve as potential candidates that can be developed into therapeutic drugs in the phytopharmaceutical industries.*

Keywords: *miswak, Persian lime, ABST, MIC, MBC Introduction*

1. Introduction

Research scientists are constantly on the lookout for innovative and breakthrough discoveries that help alleviate global enigmas, one such being the rise of antimicrobial resistance (AMR) due to overconsumption and malpractice of antibiotics, thereby endangering antibiotic efficacy and complicating the management of nosocomial infections (Pokharel and Adhikari, 2020). Consequently, AMR is associated with high morbidity; internationally, an estimated 700,000 deaths are attributed to it annually (Staa et al., 2020). Antimicrobial stewardship programs (ASP) aim to promote judicious use of antimicrobials (Akpan et al., 2020).

With declining therapeutic options, especially against widespread bacteria like *E. coli* and *S. aureus*, successful treatment remains challenging. This stimulates a growing interest in evaluating novel aspects of care, leading researchers to explore natural, non-toxic remedies derived from botanicals as a

potential resolution due to their holistic therapy, integrating mental and spiritual health (Gupta and Birdi, 2017). Since ancient times, plant extracts have not only been used to enhance flavor, aroma, color and preserve food; over 80% of the world's population, mainly India and China, use medicinal plants to combat a plethora of infections and boost immunity as they are proven to have higher efficacy and tolerance, with few to no side effects. This is due to the secondary metabolites synthesized as part of their defense mechanism (D'Souza et al., 2017). The pressing need to substitute synthetic drugs with natural alternatives is reflected by the policy imposed by the World Health Organization (WHO) promoting traditional medical practice in developing countries like Sri Lanka (Upadhyaya et al., 2017).

Miswak (*Salvadora persica*), an Arabic word meaning 'tooth-cleaning stick', is a pencil-sized stick 15 to 20 cm long with a diameter of 1 to 1.5 cm (Tatke et al., 2018). It is sourced from the roots and twigs of the Arak tree (toothbrush tree), an evergreen halophyte with a sharp taste and aromatic fragrance (Kumari et al., 2017). Due to its ethnobotanical importance, it is extensively used in the Asian, African, South American and Middle Eastern regions (particularly Islamic and Jewish communities) and is recommended for holistic oral hygiene by WHO (Albabtain et al., 2017).

Miswak holds prophylactic and therapeutic properties (Table 1). Benzyl isothiocyanate (BITC) is the main phytochemical exhibiting broad-spectrum bactericidal activity along with miswak essential oil (MEO) (Al-Bratty et al., 2020). Khalil and El-Erian (2019) noted antibiotic activity even in its gaseous form. Wrigley's company concluded mint with miswak extracts were twenty times more effective as an antibacterial (synergism), as reported by Husain and Khan (2015). Mohammed (2013) compared the extracts and various toothpastes, suggesting a possible alternative. Al-Bayati and Sulaiman (2008) and Sofrata and colleagues (2008) reported antibacterial effect against oral pathogens.

Table 1. Benefits of *S. persica* tree (Al-Bratty et al., 2020).

Plant Part	Therapeutic Uses
Roots	Increase milk production in lactating women
Leaves	Treat tooth and gum problems, stomachache, piles
Flowers	Stimulant and purgative
Bark latex	Subside skin sores
Seed oil	Treat lumbago, rheumatism, edema, malaria
Plant juice	Used as a female contraceptive

Persian lime (*Citrus latifolia*) is a thornless shrub of hybrid origin, resulting from a triploid cross between key lime (*Citrus aurantiifolia*) and lemon (*Citrus limon*), characterized by their smooth rind, seedless flesh and juiciness (Vazhacharickal et al., 2017).

Persian lime holds prolific therapeutic benefits like antimicrobial, antioxidative, anti-inflammatory, antitumor and antispasmodic properties. They are considered a nutritional powerhouse due to high content of Vitamin C, folic acid and carotenoids (Haraoui et al., 2019). Apart from being consumed worldwide as part of culinary, they are used extensively in aromatherapy and cosmetic industries (Table 2) (Bacanli et al., 2018). Being rich in bioactive phytochemicals like limonoids, coumarins and polymethoxylated flavones (PMF), limonene and beta-pinene present in lime essential oils (LEO) mainly account for the antibacterial

properties (Edogbanya et al., 2019). Berthold-Pluta and colleagues (2019) proposed using them as an alternative to synthetic preservatives. Salih (2015) studied their effects against microbes from asthma and sinusitis patients, while the use of LEO in decreasing food poisoning was proved by Jafari and colleagues (2011). The synergistic effect of lime juice in combination with herbs as a potent antimicrobial was investigated by Aibinu and colleagues (2007).

Table 2. Benefits and uses of *C. latifolia* (Bacanli et al., 2018).

Code	Sample Extracts
M/M1	Methanolic miswak 150 mg/mL
M/M2	Methanolic miswak 200 mg/mL
E/M1	Ethanolic miswak 150 mg/mL
E/M2	Ethanolic miswak 200 mg/mL
M/L1	Methanolic lime 150 mg/mL
M/L2	Methanolic lime 200 mg/mL
E/L1	Ethanolic lime 150 mg/mL
E/L2	Ethanolic lime 200 mg/mL

Since the combination involving miswak and Persian lime has not been compared previously, a novel initiative was undertaken through this study after considering their antibacterial potency and plethoric benefits, thereby aspiring to widen the market potential for herbal therapy.

2. Experimental Design

A. Study design

The present in vitro study was conducted between December 2020 to May 2021 within the laboratory premises of the Department of Biotechnology, Faculty of Science, Business Management School, Colombo, Sri Lanka. The experimental design was adapted from Edogbanya et al., 2019; Haraoui et al., 2019 and Al-Ayed et al., 2016.

Table 3. Coding for extracts.

Category	**Benefits and Uses**
Health	Heart, skin, boost immunity, digestion, iron absorption
Ayurvedic	Earache, constipation, abdominal cramps, pimples, head lice
Culinary	Lemonade, pie, garnish, pickles, alternative to vinegar
Industrial	Aromatherapy, perfume, cosmetics, soap and candle making
Domestic	Cleaning kitchen counters, cutting boards, bathroom tiles

B. Sample collection

Fresh samples of miswak twigs (imported from Pakistan) and Persian lime fruits were sourced manually from local markets in Pettah, Colombo, Sri Lanka. Strains of E. coli ATCC 25922 and S. aureus ATCC 25923 were obtained from the Medical Research Institute.

C. Sample preparation

Samples were washed with distilled water to remove unwanted debris and disinfected with 70% isopropyl alcohol; residual alcohol was left to evaporate. Samples were cut to enhance drying under shade, avoiding direct sunlight to prevent chemical modifications of the phytochemicals. They were then pulverized into a fine powder (Figure 1) and preserved through refrigeration until further use.

Figure 1. Pulverized powder of miswak twigs and lime fruits.

D. Sample extraction

Crude extraction of phytochemicals was done via cold maceration, employing polar solvents methanol and ethanol. To labelled falcon tubes, 7g of individual samples were soaked in 35 mL of 80% methanol and ethanol respectively (solid to solvent ratio 1:5). They were left on the roller mixer with continuous agitation for seven days at room temperature (RT). The solvent fraction was membrane filtered using Whatman No. 1 filter paper and concentrated through evaporation employing the fume hood for up to 48h as the solvents themselves are lethal to bacteria and residues may lead to deceptive results. The dried extracts were refrigerated at 4°C until further use. During experimentation, they were weighed and reconstituted in dimethyl sulfoxide (DMSO) to obtain working concentrations of 150 mg/mL and 200 mg/mL (Table 3).

E. Inoculum preparation

Luria-Bertani (LB) agar was used as the bacterial growth medium. The test organisms were streaked onto the agar and incubated overnight at 37°C. 5 mL Mueller-Hinton broth (MHB) was poured into two 15 mL falcon tubes. Isolated colonies of overnight primary cultures were inoculated to obtain broth cultures and incubated overnight at 37°C. The overnight cultures were diluted with distilled water and standardized to 0.5 McFarland turbidity, comparable to the optical density of a bacterial suspension with 1.5×10^8 colony forming units (CFU/mL). Fresh subcultures were prepared prior to each experiment according to the requirement of downstream procedures.

F. Antibiotic susceptibility testing (ABST)

The extracts were screened for antibacterial activity using agar well diffusion assay. Sterile petri plates were divided into quadrants and labelled. 20 mL of Mueller-Hinton agar (MHA) was poured and left to solidify. Freshly prepared inoculum cultured in MHB and standardized to 0.5 McFarland turbidity was swabbed onto the agar to achieve a confluent lawn. Wells were bored using sterile 100 µL pipette tips. The positive control (gentamicin solution for E. coli and vancomycin disks for S. aureus), negative control (DMSO) and extracts were placed in the respective wells (Table 4), sufficiently separated to avoid cross-diffusion and overlapping zones of inhibition (ZOI). The plates were allowed to stand for 15 mins at RT to ensure diffusion of the components into the agar before being incubated in an upright position overnight at 37°C. The resulting ZOI were measured and recorded. Each extract was tested against both microbes in triplicates for statistical average and reproducibility of results.

Table 4. Loading order of components.

Quadrant	Component	Conc (mg/mL)	Vol (µL)
1	P/C (CN)	1	50
	P/C (VA)	0.03	Disk
2	N/C (DMSO)	-	50
3	Sample extract	150	50
4	Sample extract	200	50

Key: P/C = positive control, N/C = negative control, CN = gentamicin, VA = vancomycin, DMSO = dimethyl sulfoxide

G. Minimum inhibitory concentration (MIC)

The MIC of lime extracts was determined to further quantitatively analyze their antibacterial potency. Samples were diluted through two-fold serial dilution (100, 50, 25, 12.5, 6.25 and 3.125%). 1000 µL sample, 900 µL MHB and 100 µL bacterial suspension were added respectively to a set of tubes, each totaling to a volume of 2 mL. The positive control contained 1700 µL of MHB, 200 µL of gentamicin and 100 µL of bacteria. The negative control contained 1900 µL of MHB and 100 µL of bacteria. The sterility control contained 2000 µL of MHB, devoid of any bacterial inoculation. A set of tubes were prepared separately for E. coli and S. aureus, sealed and incubated overnight at 37°C, following which turbidity was analyzed. The lowest concentration showing no visible growth was selected as the MIC.

H. Minimum bactericidal concentration (MBC)

A volume of 15 mL tryptone soy agar (TSA) was dispensed into petri plates under aseptic conditions. 100 µL of the macro dilution of lime extracts at concentrations above the MIC without any visible bacterial growth were spread plated until the agar surface was completely dry. Post overnight incubation at 37°C, the plate showing 99% bacterial growth arrest and its corresponding concentration was taken as the MBC.

I. Statistical analysis

The triplicate readings for ZOI were expressed as mean values (mm) ± standard deviation (SD). Two-way analysis of variance (ANOVA) and student's t-test were performed using GraphPad Prism software (version 9.1.0). Statistical significance between the antibacterial efficacy of the test samples were examined at 95% confidence interval ($p \leq 0.05$).

3. Results and Discussion

Extraction is based on solvent polarity; the degree of solubility determines the separation of constituents. Polar and nonpolar solvents yield different compositions of extracts due to differences in polarity of phytochemicals (Gonzalez-Neves et al., 2015). Other factors that affect yield include particle size, solid-solvent ratio, temperature and type and duration of extraction (Zhang et al., 2018).

The resultant percentage of yield was calculated using the following equation:

$$\text{Yield } \% = \frac{\text{Final mass of dried extract (g)}}{\text{Initial mass of raw sample (g)}} \times 100$$

According to Table 5, the highest yield was obtained for M/L (29.3%), followed by E/L (27.3%), E/M (6.7%) and lastly, M/M (4.3%). The choice of solvents in this study, methanol and ethanol, however, did not significantly impact the percentage of yield of miswak (5.5±1.2%) and lime (28.3±1.0%) extracts. Based on the student's t-test results, M/M and E/M were not statistically significant over each other at both concentrations against both bacterial strains at 95% confidence interval ($p \leq 0.05$). The results were likewise same for M/L and E/L, thereby establishing the efficacy of both solvents alike in extracting the phytochemicals.

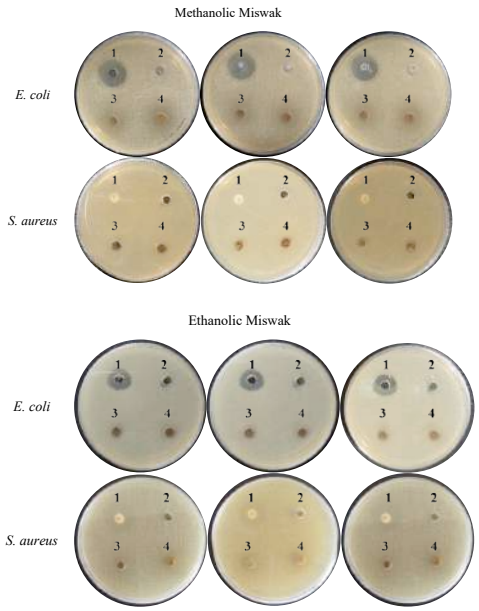
Table 5. Yield of extracts.

Sample	Mass (g)		Yield (%)	Mean ± SD (%)
	Initial	Dried		
M/M	7	0.30	4.3	5.5±1.2
E/M	7	0.47	6.7	
M/L	7	2.05	29.3	28.3±1.0
E/L	7	1.91	27.3	

Over the years, *E. coli* and *S. aureus* have been documented as notorious pathogens, exhibiting a wide repertoire of virulence

factors and antibiotic resistance (Al-Talib et al., 2016). Several studies report sensitivity of Gram-positive bacteria (*S. aureus*) to plant antimicrobial compounds. Their thick multi-layered peptidoglycan is relatively porous, permitting the passage of compounds (Turner et al., 2019). In contrast, Gram-negative bacteria (*E. coli*) possess an additional outer membrane comprising largely of lipopolysaccharides (LPS) along with a thin peptidoglycan layer. This complex cell wall structure renders them more resistant (Yamaguchi et al., 2020). Bacterial activity is affected by their growth curve; subculturing helps maintain cell viability, represented by the exponential log phase. Antibiotics that target bacterial cell wall and protein synthesis are most effective during this phase, certifying that antimicrobial action is a function of the active ingredient reaching the pathogen (Jain et al., 2020).

The ABST plates (Figure 2) for all extracts are depicted in triplicates. The wells marked 1, 2, 3 and 4 represent the positive control, negative control, 150 mg/mL and 200 mg/mL respectively.



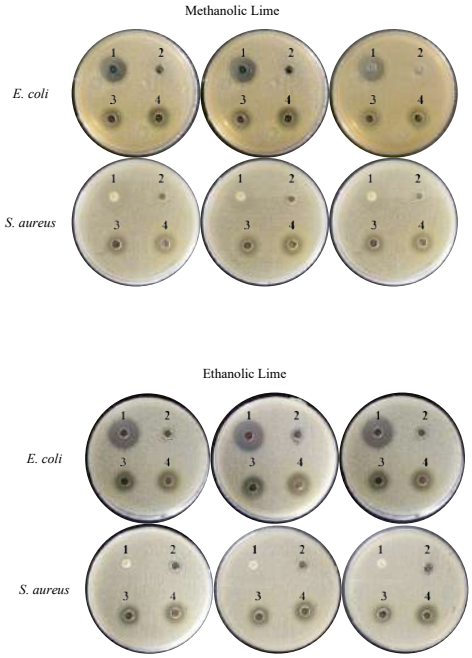


Figure 2. Well diffusion for miswak and lime extracts.

According to the ABST results, both *E. coli* and *S. aureus* exhibited susceptibility toward all extracts, but with differing degrees (Table 6). This is on par to the findings of Al-Ayed and colleagues (2016) and Aibinu and colleagues (2007), stating the antibacterial activity of miswak and lime extracts respectively. M/L2 produced the highest mean ZOI against *E. coli* (19.7±1.2 mm) while the lowest was by M/M1 against *S. aureus* (10.7±0.6 mm). The results of this study, in comparison to the positive controls, imply modest to good antibacterial activity. As per the clinical and laboratory standard institute (CLSI), gentamicin susceptibility is denoted by a ZOI above 15 mm using 10 µg/disk, while it is 17 mm using 30 µg/disk for vancomycin.

Table 6. Mean values of inhibition zones.

Extract	Mean ± SD (mm)	
	E. coli	*S. aureus*
M/M1	13.0±0.0	10.7±0.6
M/M2	13.7±0.6	12.0±1.7
P/C	20.0±0.0	20.0±1.7
E/M1	12.3±1.2	12.0±1.0
E/M2	13.0±0.0	13.0±1.0
P/C	13.3±0.6	20.7±1.5
M/L1	17.7±0.6	18.0±1.0
M/L2	19.7±1.2	19.3±1.2
P/C	19.7±1.2	20.3±0.6
E/L1	17.7±1.5	16.7±0.6
E/L2	19.3±0.6	18.0±1.0
P/C	20.0±0.0	21.0±0.0

The graphs (Figure 3) graphically compare the extract concentrations and their respective ZOI. The data represents mean (mm) ± SD for triplicates.

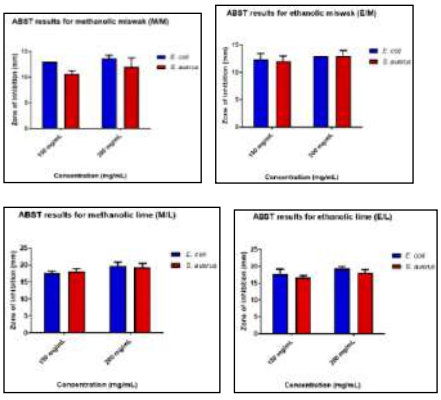


Figure 3. Comparison between miswak and lime extracts.

According to the two-way ANOVA generated, M/M demonstrated a significant difference between the bacterial strains at 95% confidence interval ($p \leq 0.05$), while no significance was identified between the concentrations. This implies it was more effective against *E. coli* than *S. aureus* at an ideal concentration of 150 mg/mL. Resistance against *E. coli* could have been overcome due to the broad spectrum of antibacterial compounds and hydrophobicity of the extracts, enabling them to break the lipid membrane and mitochondria of the bacteria. Disruption of the cell wall makes them more permeable and inhibits resistance (Rios et al., 2016). As for E/M, no significant differences were noted between the bacteria and concentrations, concluding that it was equally effective against both *E. coli* and *S. aureus* at an ideal concentration of 150 mg/mL. The activity of miswak extracts was not concentration dependent, in contrast to the findings of Khalil and El-Erian (2019). Differences in results may be correlated to geographical distribution of the plant, bacterial strains used and diffusion properties of the tested material and media (Abdallah and Al-Harbi, 2015). This study utilized MHA media throughout the qualitative susceptibility testing due to its non-differential nature. The starch absorbs toxins released by bacteria, minimizing their interference with antibiotics. Being a loose agar, it better mediates diffusion of antibiotics, leading to a truer ZOI (Mattei et al., 2014).

The outputs for M/L and E/L both indicated a significant difference between the concentrations at 95% confidence interval ($p \leq 0.05$), while neither displayed significance between the bacterial strains. This confirms that an increase in concentration from 150 mg/mL to 200 mg/mL significantly enhanced the antibacterial activity of both M/L and E/L. This reiterates that the antimicrobial activity of a substance is concentration dependent, in concordance with the report of Dubey and colleagues (2014).

The statistical analyses results are summarized in Table 7.

Table 7. Summary of results.

Sample	ANOVA		Student's t-test
	Conc. (150 and 200 mg/mL)	Bacteria (*E. coli* and *S. aureus*)	Solvents (methanol and ethanol)
M/M	–	+	–
E/M	–	–	
M/L	+	–	–
E/L	+	–	

Key: '+' indicates presence and '–' indicates absence of significant difference at 95% confidence interval ($p \leq 0.05$) between the samples and variable factors.

Since lime extracts proved their supremacy over miswak by displaying greater mean ZOI, they were further subjected to MIC and MBC assays which are complementary to each other. They allow simultaneous assessment of a test material's potency and resistance by measuring the effect of decreasing concentrations to inhibit or completely kill $1x10^6$ microbes during an 18-20h incubation period (35±2°C) (Venkateswarulu et al., 2019). These evaluations are useful during the research and development (R&D) phase of drug production (Owuama, 2017).

The MIC for M/L and E/L (Figure 4) was observed against both microbes at 12.5 mg/mL (dilution 4) based on turbidity. The sterility and negative controls (tube 7 and 8) had no visible growth in all sets, while the positive control (tube 9) was turbid, indicating bacterial growth.

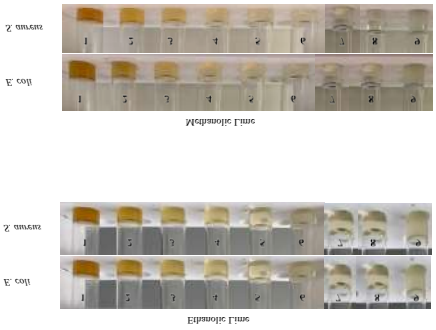


Figure 4. Minimum inhibitory concentration for lime extracts.

The MBC for M/L and E/L (Figure 5) was spotted at 25 mg/mL against both microbes, displaying 99% bacterial growth arrest. The plates marked 1 and 2 respectively represent the MBC (25 mg/mL) and MIC (12.5 mg/mL) of the extracts for relative comparison.

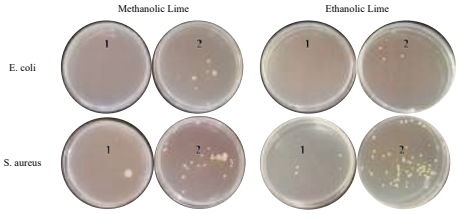


Figure 5. Minimum bactericidal concentration for lime extracts.

Antibacterial agents are regarded as bactericidal if the MBC is no more than four times the MIC (Parvekar et al., 2020). Lower the scores, the more efficacious the drugs. The results of the current study complement this with a low MIC score and a double score for MBC for both M/L and E/L, highlighting their effectiveness.

Advanced in vitro and in vivo microbiological studies involving clinical trials are necessary to standardize the inhibitory and bactericidal power of miswak and lime extracts. Herbal medicines tend to have broad synergistic effects on physiological systems which are in

the same therapeutic direction. This study can be extrapolated to inspect their synergistic compliance using the checkerboard assay and represented as a fractional inhibitory concentration (FIC) index. Knowledge of the underlying interactions between individual and combined effects is crucial to ascertain botanicals as promising antimicrobial agents.

4. Conclusion

In conclusion, the creening of medicinal botanicals contributes toward exploring novel therapeutics in an effort to eradicate the growing phenomenon of AMR. Both miswak and lime extracts exhibited antibacterial activity against *E. coli* and *S. aureus*, although lime extracts demonstrated supremacy as proven by the microbiological tests of this study. Nonetheless, based on overall results, both extracts are promising candidates for the development of therapeutic drugs in the phytopharmaceutical industries.

Acknowledgement

We would like to acknowledge every person who helped us in different ways during the research work.

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