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ORIGINAL ARTICLE

Phytochemical, Anti-inflammatory Study of the Aqueous Extract of *Rosmarinus officinalis* and *Rubia cordifolia*

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ABSTRACT
Plants are applied in the traditional healing practices from the old age as a safer and more affordable curative for chronic and incurable illnesses. In our work, chose two medicinal plants (Rosmarinus officinalis/R. officinalis and Rubia cordifolia /R. cordifolia) for preparing soap in a natural way. Phytochemical screening displayed the presence of various types of secondary metabolites in the aqueous extracts of both plants. FTIR spectral outcome disclosed the functional groups of bioactive phytometabolites. The anti-inflammatory activity of the aqueous extracts of R. officinals and R. cordifolia was evaluated. The aqueous extract of both the plants displayed dose-related anti-inflammatory activity. Even the aqueous extracts of both the plants were bactericidal towards Staphylococcus aureus, Staphylococcus haemolyticus and Bacillus cereus. The antibacterial activity was concentration dependent. Moreover, the aqueous extracts of both plants were active against Candida albicans and Trichophyton rubrum at 20 µg/mL and 30 µg/mL. The soap prepared from both the plant extracts were safer for the skin as they displayed anti-inflammatory and antimicrobial properties which may aid in protecting the skin and keep it radiant and healthy.
Keywords: *Medicinal plants, Rosmarinus officinalis, Rubia cordifolia, anti-inflammatory, antibacterial, antifungal*

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INTRODUCTION

From the emergence as well as advancement of human civilization, the medicinal plants are prioritized as the safest healer and the information on the healing benefits of medicinal plants have been passed efficaciously to the successive population or generation. The utility of various types of plants in the traditional medicinal system presents a thick bonding between the human race and the mother nature [1]. Even World Health Organization presented in their survey report that maximum percentage (80%) of the human race prominently depends on medicinal plants for health betterment. Majorly it is seen in developing nations [2]. The herbal or plant-derived medicine are more affordable, easily available and less toxic or non-toxic in contrast to the allopathic drugs [3]. Moreover, the Indian system of traditional medicine such as Siddha, Unani and Ayurveda is entirely focused on plant-based treatment of human illnesses [6]. In various formulations such as decoctions, tinctures, powders, teas and herbal infused products, the medicinal plants are used. The phytometabolites of the medicinal plants are highly potent in alleviating various health issues and also forms a raw material for formulating drugs and herbal products [4].

Inflammation is usually a natural mechanism activated in the human system as a defensive process towards the microbial pathogens (viruses, fungi and bacteria), physical/chemical irritants. This process also facilitates healing of wound. Initial inflammation (acute type) and prolonged inflammation (chronic

type) are majorly two forms of inflammations that generally happens in the living system [5]. Swelling, redness, heat, pain and edema are the characteristic of inflammatory spot usually noticed along with discomfort [1]. Repairing of damaged tissue happens through acute inflammatory reactions whereas chronic inflammatory reactions promote incurable health issues such as arthritis, diabetes mellitus, cardiovascular diseases, neurodegenerative diseases and cancer [2]. NSAIDs (non-steroidal anti-inflammatory drugs) and steroidal drugs are usually provided to control the inflammatory reactions and related damages. But these drugs represent toxic effects on the human system such as gastrointestinal ulceration, osteoporosis and hemorrhage [6-9]. The phytochemicals alkaloids, terpenoids, sesquiterpenes, steroids, curcuminoids, phenolic acids and flavonoids and their derivates are efficacious in controlling chronic inflammatory process by suppressing the release of inflammatory chemicals [10].

The resistance nature of bacterial and fungal species towards the availed antibiotics have become an unavoidable issue in the hospitals thereby creating the greater risk of mortality every year. The usage of antibiotics in an inappropriate mode has diminished the efficacy of the drugs making the infectious disease treatment more challenging [11]. Among bacteria, both gram-positive as well as gram-negative forms are highly potent of creating infections in the human and other organism [12]. Fungal infections are also highly fatal to the human life which usually occurs superficially or systematically. Most of the fungal infections are opportunistic in nature and happens in the immune-compromised individuals, patients undergone chemotherapy or blood transfusion [13]. The fungal resistance towards the antifungal medicines have made them weaker for eradicating the issue [14]. The secondary metabolites (glycosides, saponins, quinones, alkaloids, terpenoids, saponins and tannins) from the plants owns a vast spectrum of antimicrobial function [15].

Soap is produced by chemically reacting the TG (triglycerides) and the solution of lye through a process known as saponification. Generally, soaps are commercialized in liquid or solid forms and plays crucial role in skin hygiene. In markets, the commercialized soaps advertised for youthful and radiant skin are usually contains synthetic chemicals such as paraben and triclosan to add antimicrobial and anti-radical properties. but these chemicals are highly carcinogenic [16]. The herbal soaps contribute anti-radical, anti-proliferative and microbicidal properties as they contain bioactive phytometabolites and shields the skin from cracking, dryness, infections, skin cancer, eczema and keratosis [17]. In the previously done research works, various herbal infusions were used to synthesize natural soaps with distinctive aroma and natural colour [18].

Rosemary (*Rosmarinus officinalis*/ *R. officinalis*) is medicinally indispensable plant displaying nativity to Mediterranean sector and hugely cultivated world-wide. It belongs to the Lamiaceae family, perennial shrub growing up to 2 m and its leaves owns unique fragrance. Numerous phytochemicals such as rosmaquinones A and B, rosmadial, carnosol, ursolic acid, rosmarinic acid and caffeic acid have been isolated from the leaves of this plant [19]. Due to the existence of these compounds, it displays efficacious anti-radical, anti-proliferative, anti-inflammatory, wound healing and neuroprotective properties [20, 21]. *Rubia cordifolia* (*R. cordifolia*) categorized in the family of Rubiaceae is herbaceous perennial with longer, cylindrical and reddish colour rooted plant. It is vastly present in India, Africa and tropical regions of Australia [22]. Commonly in India it is recognized as Manjistha. Chemical constituents such as rubiadin, morphine, triterpenes, anthraquinones, glycosides and iridoids were identified in the various sections of *Rubia cordifolia*. All these compounds own noteworthy anti-proliferative, anti-radical and anti-inflammatory activities [23].

MATERIAL AND METHODS

Sample collection and preparation of the aqueous extract

The *R. cordifolia* and *R. officinalis* were collected from the local herbal market of Vaniyambadi, Tamil Nadu, India. Both the samples were rinsed in distilled water and dried for fifteen days. Using home mixer, powdered form of both samples was obtained. Then individually each sample was placed in the soxhlet apparatus to obtain the aqueous extract. The aqueous extract obtained was applied for phytochemical screening and to assess the anti-inflammatory and antimicrobial activities. Even the preparation of soap was done using the aqueous extract of both the samples.

Qualitative profiling of phytometabolites

The aqueous extract of *R. cordifolia* and *R. officinalis* were taken for qualitative phytochemical studies adopting the published protocols [26,2].

Carbohydrate identification

- **Molish's test:** Precisely, two milliliters of the aqueous extract of each sample were mingled gently with equal proportion of Molish's reagent and then carefully shaken. Following this, two drops of concentrated solution of sulphuric acid was placed along the sides of the test tubes. A

brownish to reddish ring development displayed carbohydrate's presence in the examined sample.

- **Benedict's test:** Exactly two millilitres of the aqueous extract was mingled with Benedict's reagent and placed in a boiling water bath. A brick red precipitate conveys reducing sugar presence.
- **Fehling's test:** Both Fehling's A as well as Fehling's B solution was mingled carefully with 2 mL of the aqueous extract of each sample in an individual test tube and placed in the boiling water bath. Reddish precipitate or brownish colour formation conveys reducing sugar existence in the sample.

a) Alkaloid identification:

- **Mayer's test:** To each aqueous extract (2 mL) taken in a separate test tube, Mayer's reagent (1 mL) was poured and mingled well. A yellowish or whitish precipitate reflected the alkaloid's existence.
- **Wagner's test:** To 1 mL of each aqueous extract, 1 mL of Wagner's reagent was added and amalgamated gently. Reddish precipitate development conveyed alkaloid in the aqueous extract.
- **Dragendorff's test:** Precisely, 1 mL of each aqueous extract is mixed well with Dragendorff's reagent. Reddish or orangish colour formation dictated alkaloids presence.
- **Hager's test:** 1mL of picric acid solution was mingled with each aqueous extract individually taken in the test tube. A yellowish tinge or colouration displayed alkaloid's presence.

b) Protein identification

- **Ninhydrin test:** A violet colouration development on heating aqueous extracts with the Ninhydrin reagent suggested evidence of protein.
- **Xanthoproteic test:** On heating aqueous extract with concentration solution of nitric acid yellowish colouration justifies amino acid presence.

c) Tannin identification

- **Vanillin-HCI test:** Exactly, 2 mL of each aqueous extract is blended gently with alcoholic solution of vanillin made by diluting 1 gram of vanillin in 10 mL of alcohol. A brick reddish colour spotted signifies condensed tannins in the aqueous extracts.

d) Glycoside identification

- **Keller-Killiani test:** Each aqueous extract (5 mL) was mingled with 2 mL of glacial acetic acid and ferric chloride (one drop made as 2% w/v) and concentrated solution of sulphuric acid. A brownish thick ring developed between the two layers of the solution dictated the cardiac glycoside entities in the aqueous extract.

e) Saponin identification: A foam appearance on vigorously shaking each phytoextract (2 mL) with 3 mL of distilled water proven saponin in the extracts.

f) Phenol identification: Two millilitres of each aqueous extract taken in the sterile test tubes was amalgamated with 2% solution of ferric chloride. A bluish or dark greenish colour proven phenol presence in the extracts.

g) Flavonoid identification: To 2 mL of each aqueous extract, five millilitres of ethanol (95%), magnesium turning (2 strips) and three drops of hydrochloric acid (concentration solution) was added and retained to react. Pinkish colouration conveyed flavonoid in the extract.

FTIR spectral study

Each phytoextract (2 mg) was blended properly with 200 mg of potassium bromide to design a pellet. The pellet was retained in the FTIR sample holder and the scanning was done from 400 cm^{-1} to 4000 cm^{-1}.

Anti-inflammatory activity of aqueous extracts

Amending the already available protocol, the anti-inflammatory activity of both the aqueous extract was studied using BSA (Bovine serum albumin) assay [19]. For this assay, precisely, 0.5 mL of each aqueous extract with varying concentrations (10 µg/mL to 50 µg/mL) was mingled with BSA solution (0.5 mL made as 0.2% maintaining a pH of 6.8 in a Tris buffer. Incubation of the solution was done for fifteen minutes at ambient condition and heated for five minutes at 72 °C in a water bath. Post heating, the content was cooled and turbidity of the protein precipitation was checked for each extract in a spectrophotometer at 660 nm. Diclofenac sodium was used as positive reference drug. The inhibition of precipitation was presented in percentage through a calculation given below:

$$\% \text{ BSA precipitation inhibition} = [(\text{Control AB} - \text{Sample AB})/\text{Control AB}] \times 100$$

Antibacterial activity of aqueous extract

Adopting agar-well diffusion protocol with required slight changes the antibacterial performance of each aqueous extract was evaluated. MHA (Muller-Hinton Agar) media was made and poured gently into the sterile perti-plates and retained for solidification. Once solidified, bacterial culture (100 µL) from each strain (*Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Bacillus cereus*. Then applying a sterilized borer well were designed in the solidified MHA media and into that poured varying doses (10 µg/mL to 40 µg/mL) of each aqueous extract. Incubation at 37 ℃ for 24 h was performed for each plate and the inhibitory zone appeared surrounding each well was noticed and measured carefully. In millimetres, the zone of inhibition developed was noted. Ciprofloxacin (10 µg/mL) functioned as positive control [27].

Antifungal activity

Implementing the agar-well diffusion methodology the antifungal performance of both the aqueous extract was checked towards the infective *Candida albicans* and *Trichophyton rubrum*. For the assay, PDA (Potato Dextrose Agar) media was made and poured gently into the petri-plates. Once the PDA media got solidified, the fungal strains were swabbed gently over the media and the wells were made using a cork-borer. Two different doses (20 µg/mL and 30 µg/mL) of each aqueous extract were placed into the wells using sterile micropipette. Exactly, ten microgram per millilitres of fluconazole drug was use for reference. All the petri-plates were placed in an incubator at 37 ℃ for 48 h [28].

Herbal soap preparation

The preparation of herbal soap using the aqueous extract of *R. cordifolia* and *R. officinalis* was done using cold process. For this, the aqueous extract of each herb (25 mL) was taken in a beaker (250 mL) and mixed with the sodium hydroxide (lye) solution prepared by dissolving 30 g in distilled water (50 mL) through heating at 70 ℃. Stirring of the solution was done while heating in the water bath until the mixture becomes thickened. The thickened mixture was carefully transferred into the silicon mould and retained for solidification for five days [3].

Statistical analysis

Each test was done thrice (n=3) and given as mean ± SD using origin software (version 8).

RESULTS AND DISCUSSION

Qualitative analysis

The phytochemical study of *R. officinalia* aqueous extract displayed the presence of alkaloids, saponins, tannins, proteins, amino acids and terpenoids. Also, the phytochemical analysis of aqueous extract of *R.cordifolia* showed the presence of alkaloids, carbohydrates, glycosides, flavonoids, amino acids, proteins and tannins.

FTIR analysis

Generally, FTIR analysis signifies the existence of phytometabolite's functional group. In the executed study, the FTIR spectral of both *R. officinalis* and *R.cordifolia* aqueous extract was done. The spectral peaks as well as bands were formed and presented in the Figure 1a and Figure 1b. The FTIR spectrum of *R. officinalis* showed peak at 344.15 cm-1, 2079.07 cm-1, 1634.52 cm-1 and 687.89 cm-1 which signified the presence of hydroxyl, carbonyl and aromatic groups. Similarly, the FTIR spectrum of *R. cordifolia* extract represented peak at 3436 cm-1, 2075.13 cm-1, 1634.95 cm-1 and 666.99 cm-1. All the appeared peaks presented the existence of hydroxyl/phenolic, amide, carboxylic and amine groups.

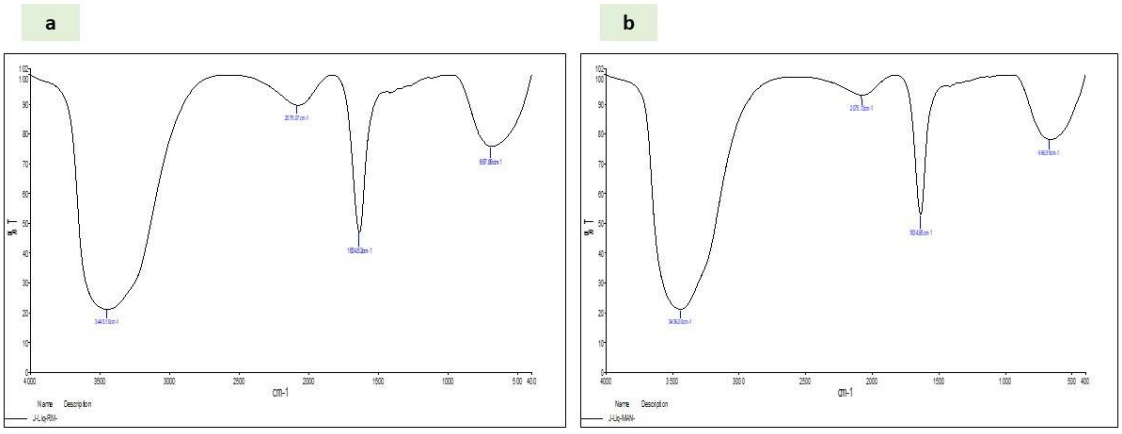

Figure.1: FTIR spectra of (a) *R. officinalis* aqueous extract (b) *R.cordifolia* aqueous extract

Anti-inflammatory activity

The ability of *R. officinalis* aqueous extract to inhibit the denaturation of bovine serum albumin protein was evaluated. At 10 µg/mL dose, 11.21% of inhibition of bovine serum albumin denaturation occurred whereas at 50 µg/mL dose, 55.11% of inhibition towards the denaturation of bovine serum albumin was noticed. The IC_{50} value of the *R. officinalis* was 16.94 µg/mL. Diclofenac comparative drug presented anti-inflammatory activity ranging from 45.61% to 84.50% in a dose-based form (**Figure.2)**. Similarly, the anti-inflammatory performance of the *R. cordifolia* aqueous extract was assessed applying varying doses. At the milder dose (10 µg/mL), the anti-inflammatory function was 9.87% and at 50 µg/mL the inflammation inhibition percentage was 36.71%. The IC_{50} dose of the *R.cordifolia* was 9.07 µg/mL. Diclofenac sodium gave 28.65% to 55.11% of inhibition towards the protein precipitation of bovine serum with IC_{50} dose of 13.65 µg/mL (**Figure.3)**. Protein (albumin) denaturation is the crucial cause of inflammatory reactions. Phytometabolites from the plants aid in inhibiting the denaturation of protein molecules induced by heat in the *in-vitro* studies [24].

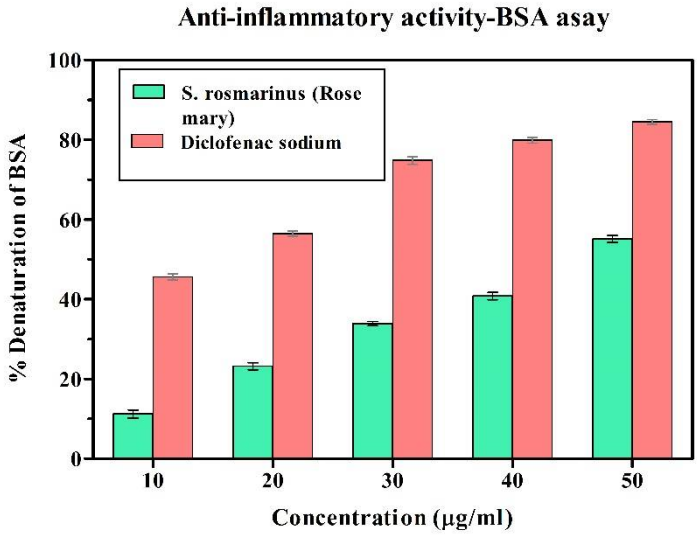

Figure.2: Presenting the anti-inflammatory activity of *R. officinalis* aqueous extract through BSA assay.

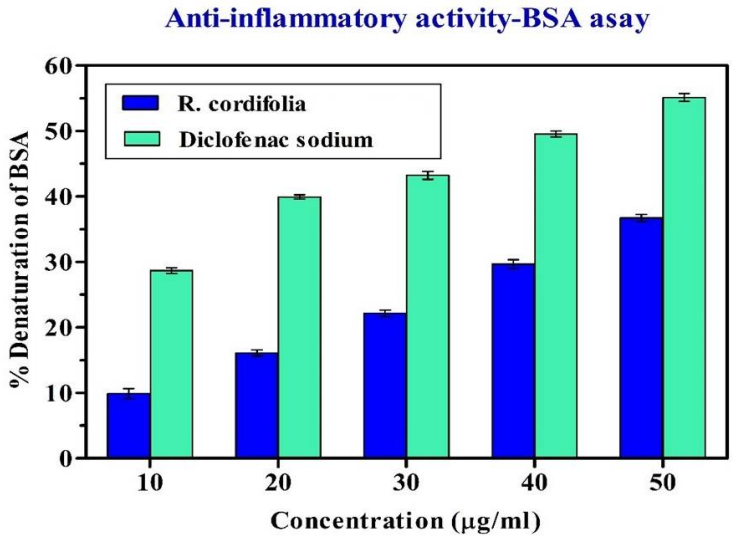

Figure.3: Presenting the anti-inflammatory activity of *R. cordifolia* aqueous extract through BSA assay.

Antibacterial activity

The antibacterial nature of *R. officinalis* aqueous extract was checked applying agar-well diffusion protocol. On analysis, it was found that as the concentration of *R. officinalis* extract raised, it's the bactericidal activity was enhanced. At 40 µg/mL, the zone of inhibition developed for the *Bacillus cereus* was 16 mm, whereas as 15 mm inhibitory zone was formed for *Staphylococcus haemolyticus*. The inhibitory zone of 14 mm was seen for *Staphylococcus aureus*. Overall, the antibacterial performance of

the *R. officinalis* extract displayed through the inhibitory zone formation was given in the Table.1. Similarly, the bacterial inhibiting performance of the *R. cordifolia* aqueous extract was evaluated. At 40 µg/mL, highest inhibitory zone width of 17 mm was appeared for *Bacillus cereus* followed by *Staphylococcus aureus* with 16 mm inhibitory zone diameter. For *Staphylococcus haemolyticus* the zone of inhibition diameter was 15 mm on treatment with the extract of *R. cordifolia*. As the dose of the *R. cordifolia* raised the width of inhibition zone was increased presenting a dose-related activity. For both the extract, Ciprofloxacin (10 µg/mL) was used as comparative drug (**Table.2)**. Damage to the plasma membrane, breaking the cell wall, inhibiting the functioning of protein/enzymes, cytoplasmic content exudation and DNA damages are the vital mechanism followed by the phytometabolites to kill the bacterial cells [25].

Table.1: Showing the zone of inhibition given by *R. officinalis* aqueous extract against the infective three bacterial strains.

Bacterial strain	Concentration				
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	Control (Ciprofloxacin) 10 µg/mL
	Zone of inhibition (ZOI)				
Staphylococcus aureus	7	9	11	12	14
Staphylococcus haemolyticus	9	10	12	14	15
Bacillus cereus	10	12	13	15	16

Table.2: Showing the zone of inhibition given by *R. cordifolia* aqueous extract against the infective three bacterial strains.

Bacterial strain	Concentrations				
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	Control (Ciprofloxacin) 10 µg/mL
	Zone of inhibition (ZOI)				
Staphylococcus aureus	11	12	13	15	16
Staphylococcus haemolyticus	9	10	12	14	15
Bacillus cereus	8	11	13	15	17

Antifungal activity

The antifungal activity of the *R. rosmarinus* aqueous extract was checked against two infectious fungal strains. At 20 µg/mL, the zone of inhibition formed towards the *Candida albicans* was 9 mm and at 30 µg/mL, 10 mm was the inhibitory zone. Similarly, at 20 µg/mL, the inhibitory zone for the *Trichophyton rubrum* was 5 mm and at 30 µg/mL, it was 7 mm by *R. officinalis* extract **(Figure.4)**. The antifungal performance of *R. cordifolia* aqueous extract was also assessed. At 20 µg/mL, there was no inhibitory zone developed for *Candida albicans* whereas at 30 µg/mL, 8 mm diameter inhibitory zone was formed. Moreover, 6 mm sized inhibitory zone was formed for *Trichophyton rubrum* by 20 µg/mL dose of *R. cordifolia* whereas at 30 µg/mL the diameter of inhibitory zone was 9 mm (**Figure.5)**. Loss of integrity of cellular membrane, damage to the DNA, inhibition of spore formation and hyphae development, creation of reactive oxygen species (ROS) and inhibition of synthesis of ergosterol, an indispensable component of cell wall are the vital mechanism conveyed to destroy the fungi by the phytometabolites [26, 29].

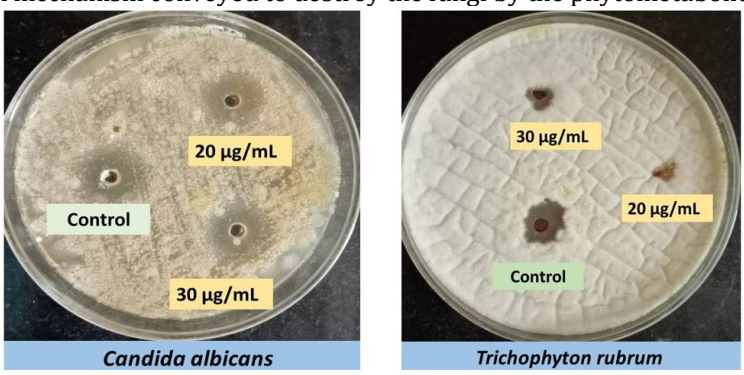

Figure.4: Presenting the antifungal images of *R. officinalis* aqueous extract against three disease creating fungal strains.

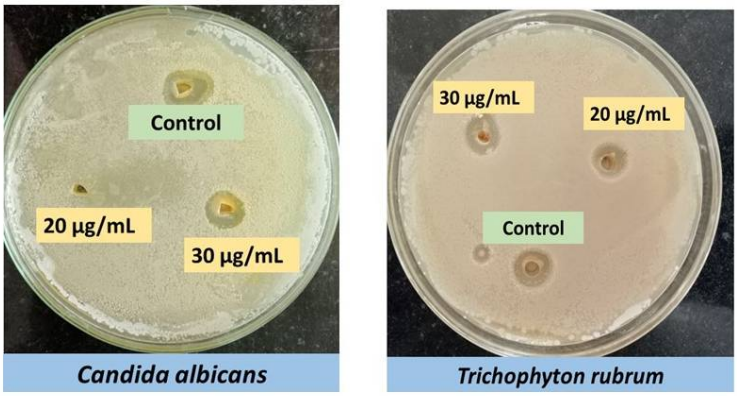

Figure.5: Presenting the antibacterial images of *R. cordifolia* aqueous extract against three disease creating fungal strains.

CONCLUSION

In our study we identified the phytometabolites in the two different medicinal plants such as *S. Rosmarinus* and *R. cordifolia* and found that both the plant extract contained the vital and medicinally valued metabolites. The FTIR unwrapped the functional groups of the phytometabolites which are crucial in displaying the bio-activities. Moreover, both the plant extracts presented anti-inflammatory, antibacterial and antifungal activity. Owned to this property both the plant extracts were applied in the preparation of natural or herbal soap which are safer and more affordable with skin benefits in comparative to the commercialized chemical soaps.

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