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FORMULATION, DEVELOPMENT AND EVALUATION OF POLYHERBAL GEL FOR EFFECTIVE TREATMENT OF ACNE

Chetan Rathore, Naveen Gupta*, Vishal Kapoor, Dr. Neeraj Sharma, Dharmendra S. Rajput

Patel College of Pharmacy, Bhopal (M.P.)

*Corresponding Author's E mail: naveenmpharm@gmail.com

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ABSTRACT

Herb is a plant or part of a plant valued for its medicinal, aromatic, or savoury qualities. Herbs can be viewed as biosynthetic chemical laboratories, producing a number of chemical compounds. Herbal remedies or medicines consist of portions of plants or unpurified plant extracts containing several constituents, which often work together synergistically. Herbal formulations have more demanded in the market. One of the most common disorders found among youngsters usually 18-25 years of age is Acne. *Acne vulgaris*, which is a skin disorder of the pilosebaceous gland leads to the formation of inflammatory lesions, seborrhea, comedone etc. The pus being formed in acne which triggers inflammation is due to Propionibacterium acnes. There is a remarkable demand of herbal formulations in the global market. *Propionibacterium acnes* are common pus-forming microbes responsible for the development of various forms of acne. In the present study anti-acne gels were prepared using polymer carbopol 940 along with the hydroalcoholic extracts of plants Seeds of *Embelia ribes* and *Piper nigrum* and evaluated for their physicochemical properties, like pH, washability, extrudability, spreadability and viscosity. The formulations (PHG1-PHG6) were tested for the anti-acne activity by well diffusion method against *Propionibacterium acnes*. Results showed that the gels were non-irritant, stable and possess anti-acne activity. The efficacy when tested with a standard was almost same to that of Clintop (Marketed gel). This suggests that Seeds of *Embelia ribes* and *Piper nigrum* has potential against acne causing bacteria and hence they can be used in topical anti-acne preparations and may address the antibiotic resistance of the bacteria.

Keywords: *Acne vulgaris, Propionibacterium acnes, Embelia ribes, Piper nigrum*, Carbopol, Physicochemical properties.

INTRODUCTION

Dried fruits of *Embelia ribes* belong to family Myrsinaceae is one of the most significant plants used from the prehistoric time in the form of the drug Baibidanga or Vidanga [1]. It has been used as an ingredient in most of the Ayurvedic formulation for the treatment of various ailments. Various

formulation of *Embelia ribes* are used in ayurvedic system of medicine like asava, aristha, lauha and taila[2]. Commonly it is known as false black pepper. It is listed in red book as threatened species. In various literatures, it is found that the fruits of that plant used as an anthelmintic, diuretic, carminative, contraceptive, anti-bacterial, anti-inflammatory astringent, antioxidant, anticancer agents and seed possessed antibiotic and antitubercular properties [3]. *Piper nigrum* belongs to the family Piperaceae, it is a perennial shrub native to southern India, and has been extensively cultivated there and in other tropical regions. As of 2013, Vietnam is the world's largest producer, as well as exporter of pepper, producing 34% of the global *P. nigrum* crop. Due to its strong pungency, it is regarded as the King of spices and it has valuable medicinal potency. It is one of the world most common kitchen spices and well known for its pungent chemical constituent piperine (1-peperoyl piperidine), discovered in 1819 by Hans Christian, which has diverse pharmacological activities. It is commonly known as Kali mirch in Urdu and Hindi, Marich in Nepali, Pippali in Sanskrit, Milagu in Tamil, and Black Pepper, Peppercorn, Green pepper, White pepper, Madagascar pepper in English [4, 5]. It is widely accepted and most used in different traditional systems of medicine, like the Unani and Ayurvedic systems [6, 7]. It has long been used to treat many diseases, such as antihypertensive, antioxidant, antiplatelets, antitumor, anticonvulsant, antithyroid, analgesic, anti-inflammatory, antidiarrheal, anti-spasmodic, antidepressants, immunomodulatory, antibacterial, antifungal, hepatoprotective, *etc.* This has lead scientists to think more about it, as a result there is much research going on regarding its derivative synthesis, SAR modification and testing its biological activities [8]. Acne vulgaris, a chronic inflammatory disease of skin, could possibly have affected almost everyone at various points in their lives [9]. The inflammatory acne lesion, a crucial event of the disease often results in scarring and permanent mark [10-13]. There is a wide range of individual clinical expression with males tending to have more severe forms, the incidence is similar in males and females until mid-20s; thereafter acne is more prevalent in females, but the severity and frequency are markedly decreased [14]. Four main pathogenetic factors of acne include hyperproliferation of follicular epithelial cells, excess sebum production and colonization of *Propionibacterium acnes* and inflammation [10, 11]. *P. acnes* have been denoted as a predominant bacterium of acne due to its unique immunomodulatory effect which mainly induces the inflammatory process [15, 16]. Skin macrophages were directly induced by *P. acnes* heat-shock protein to produce several pro-inflammatory cytokines including interleukin-6 (IL-6) and neutrophils chemoattractants; interleukin-8 (IL-8), mainly stimulates neutrophils migration leading to acne lesion and pus formation [12, 15-17]. Neutrophils subsequently generate oxygen free radicals for killing the bacteria. However, excessive production of the free radicals, stimulated by *P. acnes*, leads to the leakage of the free radicals within extracellular space, which destroys follicular epithelium and accelerates progression of the inflammatory responses [18]. Therefore, *P.acnes* has been

recognized as one of the main targets for acne treatment [9]. Nowadays, the attempts to find an alternative treatment for acne from natural resources have been considerably expanded every single year due to the antibiotic resistance of *P. acnes* and skin side effects, which might be occurred through the usage of conventional topical medicines [9, 19-22]. Ayurveda, the Indian system of medicine, has been an integral part of Indian culture and materia medica. From the rich Indian biodiversity, it has identified various plants/herbs that have been associated with a number of potential therapeutic efficacies [23].

MATERIALS AND METHOD

Materials and methods

Plant materials

Seeds of *Embelia ribes* and *Piper nigrum* were collected from local area of Bhopal (M.P.) in the month of January, 2019. Plant material (Seeds) selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder. Powdered plant material was observed for their colour, odour, taste and texture.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals and solvent used in this study were of analytical grade. The pathogenic microbes used in the current study are obtained from Microbial Culture collection, National Centre Forcell Science, Pune, Maharashtra, India.

Extraction

Dried powdered of seeds of *Embelia ribes* and *Piper nigrum* has been extracted with hydroalcoholic solvent using maceration process for 48 hrs, filtered and dried using vacuum evaporator at 40^0C and stored in an air tight container free from any contamination until it was used. Finally, the percentage yields were calculated of the dried extracts [24].

Qualitative phytochemical analysis of plant extract

The *Embelia ribes* and *Piper nigrum* extracts obtained was subjected to the preliminary phytochemical analysis following standard methods by Kokate and Khandelwal [25-26]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavanoids, glycosides, saponins, alkaloids, protein and amino acid.

Quantification of secondary metabolites

Total Phenolic content estimation

The total phenolic content was determined using the method of Olufunmiso *et al* [27]. A volume of 2 ml of extracts or standard was mixed with 1ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 1ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15min for colour development. The absorbance was measured at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/100mg).

Total flavonoids content estimation

The total flavonoid content was determined using the method of Olufunmiso *et al* [27]. 1 ml of 2% AlCl3 solution was added to 3 ml of extracts or standard and allowed to stand for 15 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100mg).

Formulating anti-acne gel

Measured quantity of methyl paraben, glycerine, polyethylene glycol and hydroalcoholic extract of seeds of *Embelia ribes* and *Piper nigrum* were dissolved in about 35 ml of water in beaker and were stirred at high speed using mechanical stirrer (or sonicator). Then carbopol 940 was added slowly to the beaker containing above liquid while stirring. Neutralized the solution by slowly adding triethanolamine solution with constant stirring until the gel is formed. All the samples were allowed to equilibrate for 24 hours at room temperature prior to performing rheological measurements (Table 1).

Table 1 Formulation of polyherbal Gel

Ingredients (%)	PHG1	PHG2	PHG3	PHG4	PHG5	PHG6
Embelia ribes extract	1gm	1gm	1gm	1gm	1gm	1gm
Piper nigrum extract	1gm	1gm	1gm	1gm	1gm	1gm
Carbopol 940	0.5mg	0.75mg	1.0 gm	1.25 gm	1.5 gm	2.0 gm
Polyethylene Glycol	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml
Methyl Paraben	0.08mg	0.08mg	0.08mg	0.08mg	0.08mg	0.08mg
Triethanolamine	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml
Distilled Water (q.s)	100ml	100ml	100ml	100ml	100ml	100ml

Evaluation of polyherbal gel

Appearance and consistency

The physical appearance was visually checked for the texture of polyherbal gel formulations.

Washability

Formulations were applied on the skin and then ease and extent of washing with water were checked manually.

Extrudability determination of formulations

The polyherbal gel formulations were filled into collapsible metal tubes or aluminium collapsible tubes. The tubes were pressed to extrude the material and the extrudability of the formulation was checked.

Determination of Spreadability

A special apparatus has been designed to study the spreadability of the formulations. Spreadability is expressed in terms of time in seconds taken by two slides to slip off from formulation, placed between, under the application of a certain load. Lesser the time taken for the separation of two slides, better the spreadability.

Method:

Two glass slides of standard dimensions (6×2) were selected. The anti-acne gel formulation whose spreadability had to be determined was placed over one of the slides. The second slide was placed over the slide in such a way that the formulation was sandwiched between them across a length of 6 cms along the slide. 100 grams of weight was placed up on the upper slide so that the anti-acne gel formulation between the two slides was traced uniformly to form a thin layer. The weight was removed and the excess of the anti-acne gel formulation adhering to the slides was scrapped off. The lower slide was fixed on the board of the apparatus and one end of the upper slide was tied to a string to which 20 gram load could be applied 50with the help of a simple pulley. The time taken for the upper slide to travel the distance of 6 cms and separate away from lower slide under the direction of the weight was noted. The experiment was repeated and the average of 6 such determinations was calculated for each anti-acne gel formulation.

$$\text{Spreadability} = \frac{m.l}{t}$$

Where, S=Spreadability (gcm/sec), m = weight tied to the upper slide (20 grams),
l= length of glass slide (6cms), t = time taken is seconds.

Determination of pH

The pH of the anti-acne gels was determined by digital pH meter. One gram of gel was dissolved in 25 ml of distilled water and the electrode was then dipped in to gel formulation until constant reading obtained. And constant reading was noted. The measurements of pH of each formulation were replicated two times.

Drug content

The drug content was determined by taking 1gm of gel in 10 ml volumetric flask diluted with methanol. 2 ml of stock solution was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15min for colour development. The absorbance was measured at 765 nm using a spectrophotometer [28-31].

***In-vitro* anti acne activity**

Preparation of plates

After sterilization, the nutrient agar in flask was immediately poured (20 ml/ plate) into sterile Petri dishes on plane surface. The poured plates were left at room temperature to solidify and incubate at 37^{o}C overnight to check the sterility of plates. The plates were dried at 50^{o}C for 30 minutes before use.

Revival of the bacterial and fungal cultures

The Bacterial cultures used in the study were obtained in lyophilized form. With the help aseptic techniques the lyophilized cultures are inoculated in sterile nutrient broth than incubated for 24 hours at 37^{o}C. After incubation the growth is observed in the form of turbidity. These broth cultures were further inoculated on to the agar plates with loop full of bacteria and further incubated for next 24 hours at 37^{o}C to obtain the pure culture and stored as stocks that are to be used in further research work.

Antibiogram Studies

The well diffusion method was used to determine the antibacterial activity of the polyherbal gel prepared from the seeds of *Embelia ribes* and *Piper nigrum* using standard procedure [32]. There were 3 concentration used which are 25, 50 and 100 mg/ml for antibiogram studies. The plates were incubated at 37^{o}C for 24 hr. and then examined for clear zones of inhibition around the wells with particular concentration of drug.

RESULTS AND DISCUSSION

The crude extracts so obtained after the maceration extraction process, extracts was further concentrated on water bath for evaporate the solvents completely to obtain the actual yield of extraction. The percentage yield of extract was given in Table 2. Phytochemical analysis of hydroalcoholic extracts of plants showed the presence of flavonoid, protein, carbohydrate and diterpines while, alkaloids, glycosides and oils and fats were reported to be absent Table 3. The determination of the total phenolic content, expressed as mg gallic acid equivalents and per 100 mg dry weight of sample. TPC of hydroalcoholic extract of seeds of *Embelia ribes* and *Piper nigrum* showed the content values of 0.675 and 0.876 respectively. The total flavonoid content of the extracts of was expressed as percentage of quercetin equivalent per 100mg dry weight of sample. The total flavonoids estimation of hydroalcoholic

extract *Embelia ribes* showed the content values of 0.223 Table 4. From the psychorheological characteristics studies of formulation showed that all of them have clear colour, No clogging, good homogeneity and smooth texture Table 5**.**

Table 2 % Yield of hydroalcoholic extract

S. No.	hydroalcoholic Extracts	% Yield (w/w)
1	*Embelia ribes* extract	4.98
2	*Piper nigrum* extract	3.55

Table 3 Result of Phytochemical screening of hydroalcoholic extracts

S. No.	Constituents	*Embelia ribes*	*Piper nigrum*
1.	Alkaloids	-ve	-ve
2.	Glycosides	-ve	-ve
3.	Flavonoids	+ve	-ve
4.	Diterpenes	-ve	-ve
5.	Phenol	+ve	+ve
6.	Amino Acids	-ve	+ve
7.	Carbohydrate	+ve	+ve
8.	Proteins	-ve	+ve
9.	Saponins	+ve	-ve
10.	Oils and fats	-ve	-ve

Table 4 Total Phenolic and Total flavonoid content

S. No.	Solvents→ Bioactive compound↓	Hydroalcoholic extracts	
		Embelia ribes	*Piper nigrum*
1.	Total Phenol (Gallic acid equivalent (GAE) mg/100mg)	0.675	0.876
2.	Total flavonoid (Quercetin equivalent (QE) mg/100mg)	0.223	-

Table 5 Results of Psycho Rheological Characteristists

Formulation	Colour	Clogging	Homogeneity	Texture
PHG1	Dark brown	Absent	Good	Smooth
PHG2	Dark brown	Absent	Good	Smooth
PHG3	Dark brown	Absent	Good	Smooth
PHG4	Dark brown	Absent	Good	Smooth
PHG5	Dark brown	Absent	Good	Smooth
PHG6	Dark brown	Absent	Good	Smooth

The results of washability, extrudability, spreadability, pH, viscosity was given in Table 6. In all formulations of gel, the spreadibility and viscocity of PHG6 is good was found to be 12.44±0.02and 3716±12. Extrudability study was performed by gel formulations were filled into aluminium collapsible tubes, the formulation has average extrudability. The skin irritation test performed showed no signs of sensitivity, erythema and edema. So the prepared formulations were considered to be non-irritant. In the all formulation of different gels the percentage of phenol content was found maximum in PHG6 Table 7.

Table 6 Results of washability, extrudability, spreadability, pH, Viscosity

Formulation	Washability	Extrudability	Spreadability (gcm/sec)	pH	Viscosity (cps)
PHG1	Good	Average	13.73±0.01	6.62± 0.11	3250±10
PHG2	Good	Average	14.65±0.01	6.75±0.15	3356±15
PHG3	Good	Average	14.15±0.02	7.55±0.01	3445±18
PHG4	Good	Average	16.65±0.01	7.15±0.13	3423±20
PHG5	Good	Average	15.12±0.02	7.24±0.12	3654±25
PHG6	Good	Average	12.44±0.02	7.01±0.12	3716±12

Table 7 Results of phenol content using Folin-Ciocalteu method

Formulation	% Phenol content
PHG1	88.11
PHG2	93.35
PHG3	87.18
PHG4	92.29
PHG5	91.16
PHG6	96.77

The efficacy of the anti-acne gels from polyherbal extracts is shown in Table 8. The anti-acne gels could inhibit the growth of the microorganisms that inhabit acnes and the ployherbal gel exhibited comparatively more efficacy to Clintop marketed gel Fig 1.

Table 8 Anti-acne activity of marketed gel and polyherbal gel formulation against *Propionibacterium acnes*

S. No.	Formulation	Zone of inhibition		
		100mg/ml	50 mg/ml	25mg/ml
1.	Clintop (Marketed gel)	18±0.5	16±0.94	15±0.57
2.	Polyherbal gel	21±0.74	19±0.86	17±0.5

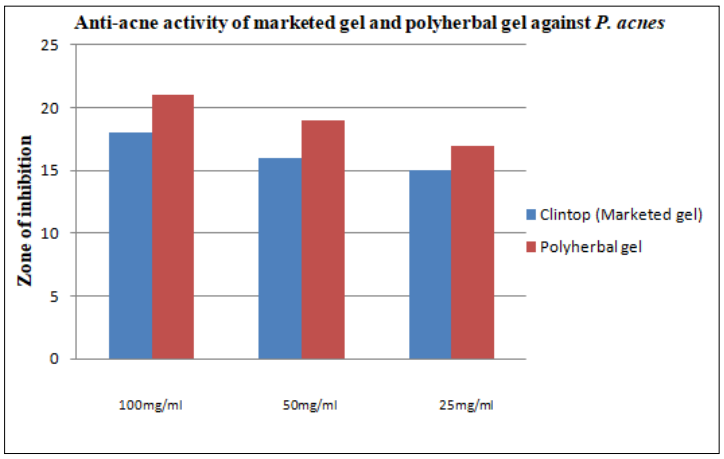

Figure 1 Anti-acne activity of marketed gel and polyherbal gel formulation against *Propionibacterium acnes*

CONCLUSION

Natural remedies are more acceptable in the belief that they are safer with fewer side effects than the synthetic ones. So, a Herbal anti-acne solution which is non-toxic, safe, effective and improves patient compliance by the utilization of herbal extracts would be highly acceptable. Polyherbal formulation contains seed of *Embelia ribes* and *Piper nigrum* passes more anti acne activity against marketed formulation. Further phytochemical studies are also required to isolate and characterize active ingredients that are responsible for its anti acne activity and to explore the existence of synergism if any, among the compounds.

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REFERENCES

1. Ambati S, Jyothi V, Jyothi A. Int. J. Pharm. Tech. 2010; 2: 525- 539.
2. Lal B, Mishra N. Int. J. Pharm. Sci. Res. 2013; 4: 3823-3838.
3. Shelar R, Maurya C, Tekale P, Katkar K, Naik V, Suthar A and Chauhan VS. Int. J. Pharm. Clin. Res. 2009, 1, 146-149.
4. Damanhouri ZA and Ahmad A. A review on therapeutic potential of *Piper nigrum* L. (Black Pepper): The King of Spices. Medicinal and Aromatic Plants. 2014; 3(3): 1-6.
5. Meghwal M and Goswami TK. *Piper nigrum* and piperine: An Update. Phytotherapy Research. 2013; 27:1121-1130.

6. Park UH, Jeong HS, Jo EY, Park T, Yoon SK, Kim EJ, Jeong JC and Um SJ: Piperine, a component of Black Pepper, inhibits adipogenesis by antagonizing PPARγ activity in 3T3-L1 Cells. J. Agrc. Food Chem. 2012; 60: 3853-3860.
7. Ahmad N, Fazal H, Abbasi BH, Farooq S, Ali M and Khan MA: Biological role of *Piper nigrum* L. (Black pepper): A review. Asian Pacific Journal of Tropical Biomedicine. 2012; 1945-1953.
8. Joshi DR, Shrestha AC and Adhikari N. A review on diversified use of the king of spices: *piper nigrum* (black pepper). International Journal of Pharmaceutical Sciences and Research 2018; 9(10): 4089-4101.
9. Webster GF. Acne. In: Callen JP. Current problems in dermatology. 1st ed. Philadelphia, Pennsylvania: Mosby A time mirror company. 1996; 238-268.
10. Layden JJ. New understandings of the pathogenesis of acne, J. Am. Acad. Dermatol. 1995; 32: 15-25.
11. Cunliffe WJ. The sebaceous gland and acne 40 years on, Dermatol. 1998; 196: 9-15.
12. Farrar MD and Ingram E. Acne: Inflammation, Clin. Dermatol. 2004; 22: 380-384.
13. Williams HC, Dellavalle RP, Garner S. Acne vulgaris. Lancet. 2012; 379: 361-372.
14. Sams WM and Lynch PJ. Principle and Practice of Dermatology. 2nd ed. New York: Churchill Livingstone.1996; 801-809.
15. Bojar RA and Holland KT. Acne and *Propionibacterium acnes*, Clin. Dermatol. 2004; 22: 375- 379.
16. Dessionioti C and Katsambas AD. The role of *Propionibacterium acnes* in acne pathogenesis: facts and controversies, Clin. Dermatol. 2010; 28: 2-7.
17. Kim J, Ochoa MT, Krutzik SR, Takeuchi O, Uematsu S, Legaspi AJ, et al., Activation of Toll-like receptor 2 in acne triggers inflammatory cytokine responses, J. Immunol. 2002; 169: 1535-1541.
18. Akamatsu H and Horio T. The possible role of reactive oxygen species generated by neutrophils in mediating acne inflammation, Dermatol. 1998; 196: 82-85.
19. Chomnawang MT, Surassmo S, Nukoolkarn VS and Gritsanapan W. Antimicrobial effects of Thai medicinal plants against acne-inducing bacteria, J. Ethnopharmacol. 10 (2005) 330-333.
20. Eady EA, Gloor M and Leyden JJ. *Propionibacterium acnes* resistance: a worldwide problem, Dermatol. 2003; 206 (1): 54-56.
21. Tsai TH, Wu WH, Tseng JT, Tsai P. *In vitro* antimicrobial and anti- inflammatory effects of herbs against *Propionibacterium acnes*, Food. Chem. 2010; 119: 964– 968.
22. Kosmadaki M and Katsambas A. Topical treatment for acne, Clin. Dermatol. 2017; 35: 173 –178.
23. Chatterjee A and Pakrashi SC. Treatise of Indian Medicinal Plants; CSIR Publication: New Delhi, 1995; Vol. I, p 26.

24. Mukherjee PK. Quality control of herbal drugs. 2nd Ed. Business Horizons; 2007.
25. Kokate CK. Practical pharmacognosy, 4th Ed. Vallabh Prakashan; 2011.
26. Khandelwal KR, Practical pharmacognosy, technique and experiments.23rdEd. Nirali Prakashan; 2005.
27. Olufunmiso OO and Afolayan AJ. phenolic content and antioxidant property of the bark extract of *Ziziphus mucronata* willd. Subsp. mucronata willd. BMC Complement Altern Med. 2011; 11:130.
28. Barry BW. Dermatological Formulations, Marcel Dekker., Inc., New York, Basel. 1983; 18: 96-115.
29. Jain S, Padsalg BD, Patel AK and Moale V. Formulation development and evaluation of fluconazole gel in various polymer bases. Asian J Pharm. 2007; 1(8):63-68.
30. Lachman L, Lieberman HA and Kanig JL. The Theory and practice of Industrial Pharmacy, Varghese publishing House, 3rd edition, 534.
31. Schoch TJ. Effects of freezing and cold storage on pasted starches. In: Tressler DK, Van Arsdel WD, Copley MJ, eds. The Freezing Preservation of Foods. Westport: CT. 1968; 4.: 44–56.
32. Bauer AW, Kirby WMM, Sherris JC and Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 1966; 45:493–496.